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Copper deficiency in rodents alters dopamine β-mono-oxygenase activity, mRNA and protein level

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Cu is an essential cofactor for at least twelve mammalian enzymes including dopamine β-mono-oxygenase (DBM), which converts dopamine (DA) to noradrenaline (NA). Previous studies reported that certain Cu-deficient (Cu−) rat tissues have lower NA and higher DA than Cu-adequate (Cu+) tissues, suggesting that DBM function was impaired. However, *in vitro* studies suggested that DBM activity is higher in Cu− tissue. Experiments were conducted on adrenal glands (AG), medulla oblongata/pons (MO), vas deferens (VD) and heart (HT) from a single rat experiment to provide data to help clarify this puzzling contradiction. *In vitro* DBM activity assays showed Cu− samples had significantly higher activity than Cu+ samples in both AG and MO, but not VD. Activity data were confirmed by Western immunoblots. Quantitative real-time PCR demonstrated higher DBM mRNA in Cu− tissues but unaltered levels of several other cuproenzymes and Cu-binding proteins. Previous pharmacological data implied that high DBM was associated with low NA. HPLC analyses confirmed that NA and DA levels in Cu− MO, VD and HT were significantly lower and higher, respectively, than in Cu+ tissues. However, the NA content of AG was not statistically lower. Furthermore there was no correlation between higher DBM mRNA and lower NA in four Cu− tissues. Adequate dietary Cu is essential to support DBM function *in vivo* but additional studies are needed to determine the mechanism for increased DBM transcription associated with Cu deficiency.

Copper deficiency: Rats: Mice: Dopamine β-mono-oxygenase: Noradrenaline

Cu was recognised as an essential nutrient after careful laboratory studies in rats and field observations in sheep and cattle in the 1920 s and 1930s[1]. As for other essential metals, Cu is required during perinatal development of the central nervous system[2]. Cu's importance for brain development was further exemplified by research done on Cu-deficient guinea-pigs where underdeveloped cerebella and hypomyelination were evident[3]. In humans, the brain requirement for Cu is best illustrated by Menkes' disease, an X-linked inherited neurodegenerative disorder which was first connected with Cu deficiency in 1972[4]. The molecular reasons for the central nervous system phenotype accompanying Cu deficiency remain unknown.

Humans require Cu as an essential catalytic cofactor for approximately twelve mammalian cuproenzymes[5]. One is dopamine β-mono-oxygenase (*EC* 1.14.17.1; DBM) that catalyses the hydroxylation of dopamine (DA) to noradrenaline (NA) using Cu, oxygen and ascorbic acid as additional substrates[6]. DA and NA are important neurotransmitters required for the proper function of the brain. DBM is located in granulated vesicles of both sympathetic nerve terminals, adrenal medulla chromaffin cells, and noradrenergic and adrenergic neurons of brain[7]. The importance of DBM was demonstrated by the embryonic lethality of DBM knockout mice[8]. *In vivo* limitation of DBM following Cu deficiency was first demonstrated in heart tissue in 1967[9]. Normally NA synthesis is limited by tyrosine mono-oxygenase activity; however, under certain circumstances DBM can be rate limiting[10].

The Cu–DBM brain connection was concurrently made 2 years after the first report that Menkes' disease was associated with aberrant Cu metabolism. These studies in brindled mice, a genetic homologue of Menkes' disease, and in Cu-deficient rats both reported lower brain NA and brain Cu[11,12]. More recently, it was observed that several brain regions had lower NA levels but higher DA levels in Cu-deficient rats and mice compared with Cu-adequate rodents[13,14]. Supported by these catecholamine results DBM activity was suggested to be lower in Cu-deficient rodent brain.

Conversely, direct *in vitro* DBM activity assay of mutant mouse brain showed an increase in DBM activity[15,16]. Additionally, following dietary Cu deficiency in both rats and mice, higher brain DBM activity was reported[13,16]. Mutant blotchy mice, another mottled mutant, also exhibited higher DBM activity in adrenal glands[15]. Young male rats following perinatal Cu deficiency also demonstrated

Abbreviations: CCS, Cu chaperone for superoxide dismutase; Cu+, Cu adequate; Cu−, Cu deficient; DA, dopamine; DBM, dopamine β-mono-oxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NA, noradrenaline; PNGase, peptide:N-glycosidase; qRT, quantitative real-time.

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higher adrenal DBM activity[13]. DBM activity should be lower based on catecholamine levels; however, using enzyme assay, DBM activity was higher after Cu deficiency. Thus, a paradox exists.

Increased DBM activity following Cu deficiency is probably due to increased DBM protein abundance rather than augmented levels of inhibitors or lower levels of activators, but this has not been rigorously investigated. Adrenal DBM protein levels in older Cu-deficient rats were higher than in Cu-adequate rats[17]. Additionally, it was reported that adrenal mRNA for DBM was also increased following Cu deficiency[18]. Medulla/pons DBM mRNA was higher in young Cu-deficient females but not males based on Northern blot data, adding further to the confusion[18]. Thus it is still unknown whether an increase in DBM protein in brain of Cu-deficient rats accompanies higher DBM activity.

The purpose of these experiments was to extend earlier observations on brain and adrenal DBM, measuring enzyme activity, mRNA, protein and catecholamine levels in a single perinatal Cu deficiency rat experiment. Additionally, a tissue innervated with sympathetic nerves, the vasa deferentia, was studied. Rat and mice adrenal NA levels are different when Cu-deficient animals are compared with Cu-adequate animals. It was not known if DBM protein levels changed in a similar manner; thus some analyses on Cu-deficient mice were conducted. A second purpose of these studies was to test the hypothesis that depletion of NA was associated with increased transcription of DBM mRNA. Prior studies by several groups using reserpine to deplete catecholamines had made this prediction[19,20].

Experimental methods

Animal care and diets

Holtzman rats and Hsd:ICR (CD-1) outbred albino mice were purchased commercially (Harlan Sprague–Dawley, Indianapolis, IN, USA). Rodents were maintained on Cu-adequate (Cu+) or Cu-deficient (Cu−) dietary treatment consisting of a Cu-deficient modified AIN-76A diet (Teklad Laboratories, Madison, WI, USA) that contained 0·36 mg Cu/kg by analysis. All dams, minimum of five litters per treatment group, and offspring were fed the Cu− diet, but Cu+ groups drank water supplemented with cupric sulfate (20 mg Cu/l), and Cu− groups drank deionised water. Cu deprivation was started at embryonic day 7 for the rats and at birth (postnatal day 0) for mice. Litter size was culled to ten pups on postnatal day 2[21]. All animals were maintained on a 12 h light cycle (07.00 to 19.00 hours) at 24°C with 55 % relative humidity and had free access to diet and water. The University of Minnesota Animal Care Committee approved all protocols.

Both male rats (postnatal day 24–postnatal day 26) and male mice (postnatal day 27–postnatal day 28) were weighed before anaesthetised with diethyl ether. The rodents were killed by decapitation. Medulla oblongata/pons, remainder of brain, liver samples, hearts, adrenal glands and vasa deferentia were harvested, weighed and processed for biochemical analysis or frozen in liquid N_2 and stored at −75°C until used.

Biochemical analyses

Selected tissues and diet were wet-digested with concentrated HNO_3 (Trace Metal grade; Fisher Scientific, Pittsburgh, PA, USA). Digested material was suspended in 0·1 M-HNO_3 and analysed for total Cu content by flame atomic absorption spectroscopy (model 1100B; Perkin-Elmer, Norwalk, CT, USA). Protein levels of tissue samples were measured using a modified Lowry method[22].

DBM activity was determined by a modified spectrophotometric method previously described[16]. Adrenal, medulla/pons and vasa deferentia tissues were homogenised in 49, 9 or 24 volumes of 5 mM-potassium phosphate (pH 7·0) containing 0·2 % Triton X-100. Conversion of tyramine to octopamine was determined as described elsewhere[23].

Analysis of dopamine β-mono-oxygenase mRNA expression

Total RNA was extracted from frozen samples using a TRI reagent® kit (Ambion, Austin, TX, USA), following manufacturer recommendations, including all optional steps. The purity of RNA was established spectrophotometrically and by RNA gels[18]. Contaminating DNA was removed using a DNA-free™ kit (Ambion), following manufacturer recommendations and cDNA was synthesised using Omniscript® Reverse Transcriptase (Qiagen®, Valencia, CA, USA) and amplified with a SYBR Green I kit (Roche, Indianapolis, IN, USA).

Copy number of rat tissue mRNA of DBM, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and/or ribosomal 18S, both used as controls, was determined by quantitative real-time (qRT)-PCR. Primers for DBM and GAPDH are listed in Table 1. Rat 18S primer pairs were 5′ CTG TGA TGC CCT TAG ATG TCC 3′ (forward) and 5′ GCT TAT GAC CCG CAC TTA CTG 3′ (reverse). Mass of the transcripts was determined by comparison with gel purified PCR product standards. Molecular weight was determined by entering full DNA sequence including the forward and reverse primers into a molecular-weight calculator to determine the complementary strand, in addition to the genes' molecular weight[24]. Dividing the mass of the gene transcript by its molecular weight and multiplying it by Avogadro's number calculated copy number.

Dopamine β-mono-oxygenase antibody characterisation

DBM antisera were developed in rabbits (Sigma Genosys, The Woodlands, TX, USA) against KLH-peptide, N-SEPPESPFPYHIPLD-C, corresponding to amino acid residues 43–57 and 44–58 in rats and mice, respectively. Anti-DBM antibody was affinity purified from final bleed serum using a SulfoLink® kit (Pierce, Rockford, IL, USA) according to manufacturer recommendations. The affinity purified DBM antibody was diluted 1:500 for Western blot analysis.

Antibody specificity was determined using two approaches, antigen blocking and mobility shift upon removal of carbohydrate. Blocking of the antibody was accomplished using Rat N-term DBM peptide (SEPPESPFPYHIPLD) with DBM primary antibody diluted 1:500 with either 12 μg of peptide or 12 μg of bovine serum albumin, as a control. Carbohydrate removal from DBM protein was accomplished

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Table 1. Relative mRNA expression of copper metabolism-related genes in medulla/pons of male rats aged 25 d (copy number per 1000 glyceraldehyde 3-phosphate dehydrogenase)†

(Mean values (n 5) with their standard errors)

Gene	Primer pair	Cu-adequate		Cu-deficient	
		Mean	SEM	Mean	SEM
DBM	Forward: TGG AAT CTT GGA GGA GAT GTG CGT Reverse: TGC CGA ACC GGT TTA CTA TGT GGA	1·83	0·08	2·76*	0·30
CCS	Forward: GAG TTG TCA GAG CTG CGT GCA C Reverse: CCC TTG AGT ACA GCC TGC CTC	37·3	3·29	36·2	6·78
COX I	Forward: TCA CTG CCA GTA TTA GCA GCA GGT Reverse: TCT GGG TGG CCG AAG AAT CAG AAT	4814	555	5879	299
COX IV	Forward: GCA CAT GGG AGT GTT GTG AAG AGT Reverse: TTT CTC ATC CCT GGA AAG GCT GCT	4222	583	5243	684
CTR1	Forward: GGA GAA ATG GCT GGA GCT TTT Reverse: CGG GCT ATC TTG AGT CCT TCA	9·79	2·13	13·3	1·82
PAM	Forward: CAC TTC GAC ATG CCT CAT GAT Reverse: TCA CAG GCA CCA AGA GT	8·09	0·51	7·55	0·16
SOD1	Forward: GCG TCA TTC ACT TCG AGC AG Reverse: ATA GGG AAT GTT TAT TGG GCA ATC	521	35·5	493	30·4

DBM, dopamine β-mono-oxygenase; CCS, Cu chaperone for superoxide dismutase; COX I, cytochrome c oxidase subunit 1; COX IV, cytochrome c oxidase subunit 4; CTR1, Cu transporter (SLC31A1); PAM, peptidylglycine α-amidating mono-oxygenase; SOD1, Cu,Zn superoxide dismutase.

* Mean value was significantly different from that of the Cu-adequate group ($P<0·05$; Student's t test).

† Genes and their respective forward and reverse primers were designed for real-time PCR. Copy numbers of specific mRNA were determined by quantitative real-time PCR and compared in Cu-adequate and Cu-deficient rats.

using peptide:N-glycosidase (PNGase) F (New England Bio-Labs, Ipswich, MA, USA) according to manufacturer recommendations.

Western blot analysis

Samples for Western blot analysis of DBM, actin and Cu chaperone for superoxide dismutase (CCS) were prepared by homogenising frozen rat adrenal gland, medulla/pons and vasa deferentia, and mouse adrenals and vasa deferentia in 99, 9, 24, 39 or 9 volumes, respectively, of 50 mM-potassium phosphate buffer (pH 7·0) containing 0·2 % Triton X-100 and protease inhibitors (Protease Inhibitor Cocktail; Sigma Chemical, St Louis, MO, USA). Homogenates were centrifuged at 10 000 ***g*** for 10 min at 4°C and supernatant fractions were saved for analysis at −75°C. Fractionation was completed on 10 % SDS-PAGE gels. Transfer of protein to 0·2 μm nitrocellulose membranes and processing for immunoblotting was described previously[17]. Membranes were stained with Ponceau S (Sigma Chemical) to verify equal protein loading.

Protein levels of CCS were evaluated using affinity purified rabbit anti-hCCS characterised previously, at a 1:500 dilution[25]. Membranes were also probed for actin to verify equal loading of protein. Chemiluminescence was captured using high-speed blue X-ray film (Lake Superior X Ray Inc., Duluth, MN, USA) and densitometry was carried out using a Kodak Image Station 2000M and Molecular Imaging Software (version 4.0.4; Kodak, New Haven, CT, USA).

Catecholamine analysis

Catecholamines were extracted from tissues by homogenising with nine volumes of 0·05 M-$HClO_4$ containing 0·3 μM-3,4-dihydroxybenzylamine[26]. Catecholamines were eluted from alumina with 200 μl of a 60:40 mixture of 0·2 M-acetic acid and 0·04 M-H_3PO_4[27]. Separation of NA, DA and 3,4-dihydroxybenzylamine was accomplished using reverse-phase ion pairing HPLC with electrochemical detection using a mobile phase of 0·5 mM-1-octanesulfonic acid, 0·1 mM-EDTA and 0·1 M-KH_2PO_4 with 6·5–8 % methanol. The pH was adjusted to 3·0 with 0·1 M-phosphoric acid. Samples were separated on a 3·2 × 15 mm cartridge guard column and a 4·6 × 250 mm analytical column (ODS-II 5 μm; Regis, Morton Grove, IL, USA). Output was recorded and peaks were integrated using Peak Simple Software (Chrom Tech, Inc., Apple Valley, MN, USA)[21].

Statistical analysis

Mean values with their standard errors were calculated. Student's unpaired two-tailed t test was used to compare data between the two dietary treatments, $\alpha = 0·05$ and $\alpha = 0·01$. Variance equality was evaluated by the F test. All data were processed using Microsoft Excel™ (Redmond, WA, USA). Immunoblot data normalisation was accomplished by assigning a value of 1·0 to the mean pixel density of the Cu+ samples. All individual Cu+ and Cu− density values were then recalculated before graphing for ease of comparison. Correlation analysis for scatter plot was calculated using Excel™ and a Pearson product moment correlation coefficient table of critical values, for two-tailed test and $\alpha = 0·05$.

Results

Rodent biochemical characteristics

Following perinatal Cu deficiency, Cu status in the rat experiment was analysed by evaluating a number of characteristics: body weight, cardiac hypertrophy (heart:body weight ratio), brain Cu and liver Cu (Table 2). All of the characteristics

Table 2. Characteristics of male Holtzman rats (aged 24–26 d) and male CD-1 mice (aged 27–28 d) following copper deficiency (Mean values with their standard errors for five to fifteen animals)

		Body weight (g)		Heart:body weight ratio (mg/g)		Brain Cu (μg/g)		Liver Cu (μg/g)	
Species	Diet	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Rat	Cu+	83·3	1·8	5·11	0·14	2·30	0·06	6·51	0·55
Rat	Cu−	61·9**	1·1	12·8**	0·64	0·44**	0·04	0·42**	0·03
Mouse	Cu+	21·6	1·1	5·30	0·11	2·99	0·12	4·28	0·29
Mouse	Cu−	19·3	1·1	8·17**	0·47	0·70**	0·06	1·22**	0·04

Cu +, Cu-adequate; Cu −, Cu-deficient.
** Mean value was significantly different from that of the Cu + animals of the same species ($P<0·01$; Student's t test).

showed a statistical difference between the Cu− and Cu+ animals. Body weights in the Cu− rats were significantly lower than in the Cu+ rats. Cardiac hypertrophy occurred in Cu− rats compared with Cu+ rats, as evident by the 2·5 times greater heart:body weight ratio. Cu− rats had a major reduction in brain Cu compared with Cu+ rats (81 %) consistent with neuronal Cu deficiency. Cu deficiency in a peripheral tissue was evident as well. Cu− rats showed a severe reduction in liver Cu compared with Cu+ rats (94 %). These four characteristics demonstrate that the dietary model in the rats studied was successful in achieving rats of two different Cu states. The same four characteristics were measured in the mouse experiment at postnatal day 27-postnatal day 28 (Table 2). Results were similar to those of the rat, except that there was no change in body weight due to Cu deficiency. Compared with Cu+ mice, Cu− mice had a heart:body weight ratio 1·5 times higher, a 77 % reduction in brain Cu and a 71 % reduction in liver Cu.

Dopamine β-mono-oxygenase antibody specificity

Two experiments were performed to determine DBM antibody specificity. Affinity purified DBM antibody detected a single immunoreactive band of approximately 75 kDa in adrenal glands from rats and mice, similar to the size previously characterised[28]. The antibody detected multiple immunoreactive bands for medulla oblongata/pons, all smaller in size than 75 kDa (Fig. 1). In experiment 1, two Western blots with adrenal glands, medulla oblongata/pons, and liver, as a negative control, were performed: one in which the antibody was treated with bovine serum albumin and one treated with DBM blocking peptide (Fig. 1 (a)). Antibody treated with bovine serum albumin results in a single adrenal gland band (75 kDa) and multiple medulla oblongata/pons bands. The lane loaded with liver had no immunoreactive band. In contrast, the blot treated with DBM peptide clearly shows that the band at 75 kDa in the adrenal gland lane and the slightly smaller band in the medulla oblongata/pons lane are blocked by DBM peptide.

In experiment 2, a Western blot was performed with adrenal gland and medulla oblongata/pons samples. Cu− samples indicated by (−) and Cu+ samples (+) above the blot were compared (Fig. 1 (b)). Samples before loading were either treated with PNGase (+) or just buffer (−). The adrenal gland sample not treated with PNGase displayed the usual immunoreactive band detected at 75 kDa. In contrast, the adrenal gland sample treated with PNGase migrated further, due to the removal of carbohydrate from DBM. Both the Cu− and Cu+ medulla oblongata/pons samples yielded a pattern approximately the same as before (Fig. 1 (a)), when not treated with PNGase (Fig. 1 (b)). The upper band in both Cu− and Cu+ medulla oblongata/pons samples disappeared when treated with PNGase. The second band was more dense when treated with PNGase. This is consistent with the removal of carbohydrates from DBM and a similar migration shift of DBM seen for adrenal glands.

Adrenal gland analysis

Adrenal gland homogenates from postnatal day 24 male rats were used to determine DBM activity which was greatly influ-

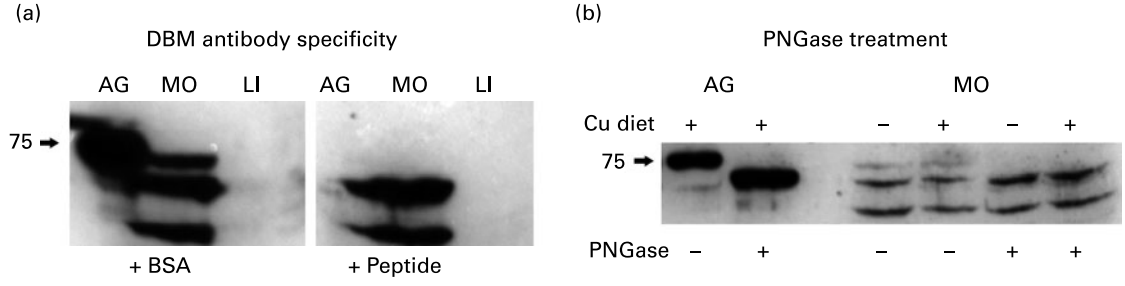

Fig. 1. Dopamine β-mono-oxygenase (DBM) antibody characterisation. (a) Blocking of antibody with DBM peptide or bovine serum albumin (BSA) was analysed by Western blot of adrenal glands (AG) and medulla oblongata/pons (MO). Liver (LI) was used as a negative control. (b) Cu-adequate (+) or Cu-deficient (−) samples were either treated with peptide:N-glycosidase (PNGase) (+) or buffer (−) and were analysed by Western blot. DBM bands in both the AG and MO lanes migrated further down the blot when treated with the PNGase, consistent with carbohydrate removal.

enced by Cu deficiency. Activity, *in vitro*, of DBM from Cu− adrenal gland samples was 2·4-fold higher than that of Cu+ samples (Fig. 2 (a)).

Total RNA from adrenal glands was isolated from postnatal day 25 male rats of both treatment groups and qRT-PCR was performed to determine the copy numbers of DBM and GAPDH, an expression control. The number of copies of DBM transcript per 1000 copies of GAPDH transcript was significantly higher, 0·7-fold, in Cu− compared with Cu+ samples (Fig. 2 (b)). GAPDH copy number was not affected by diet.

To extend data for DBM activity and transcript levels, adrenal gland DBM protein abundance of postnatal day 24 male rats was measured by Western immunoblot. The DBM immunoreactive band is clearly detected at 75 kDa. It was apparent that DBM protein abundance was markedly higher in Cu− than the Cu+ samples. Cu status was determined by measuring CCS protein abundance. CCS abundance has been shown to increase when Cu is limiting[29]. In adrenal glands CCS abundance was markedly higher in Cu− than Cu+ tissue (Fig. 2 (c)). Actin, a loading control, was not changed by treatment (Fig. 2 (c)). Mean DBM density of Cu− adrenal glands was 1·7-fold higher than that of the Cu+ samples (Fig. 2 (d)). Mean CCS density in Cu− adrenal glands was even higher, a 3·6-fold difference (Fig. 2 (d)).

Medulla oblongata/pons analysis

DBM activity in medulla oblongata/pons from postnatal day 24 male rats was greatly influenced by Cu deficiency and was 0·9-fold higher in Cu− medulla oblongata/pons than Cu+ medulla oblongata/pons (Fig. 3 (a)). Specific activity in medulla oblongata/pons was much lower than for adrenal glands.

Total RNA from medulla oblongata/pons was isolated from postnatal day 25 male rats of both treatment groups and qRT-PCR was performed to determine the copy numbers of both DBM and GAPDH. DBM transcript abundance, per 1000 copies of GAPDH, was significantly higher, 0·5-fold, in Cu− compared with Cu+ rats (Fig. 3 (b)). GAPDH copy number was not different between the two groups. There was very low expression of DBM mRNA in medulla oblongata/pons compared with adrenal glands. In Cu+ rats, abundance in medulla oblongata/pons was less than 2 per 1000 GAPDH, whereas in adrenal glands, abundance exceeded 250. This fact made Western immunoblot detection of medulla oblongata/pons DBM very challenging as was shown in Fig. 1.

Medulla oblongata/pons DBM protein abundance of postnatal day 26 male rats was measured by Western immunoblot and the immunoreactive DBM band was detected slightly below the 75 kDa marker. It was apparent that both DBM and CCS protein abundance was higher in Cu− than Cu+

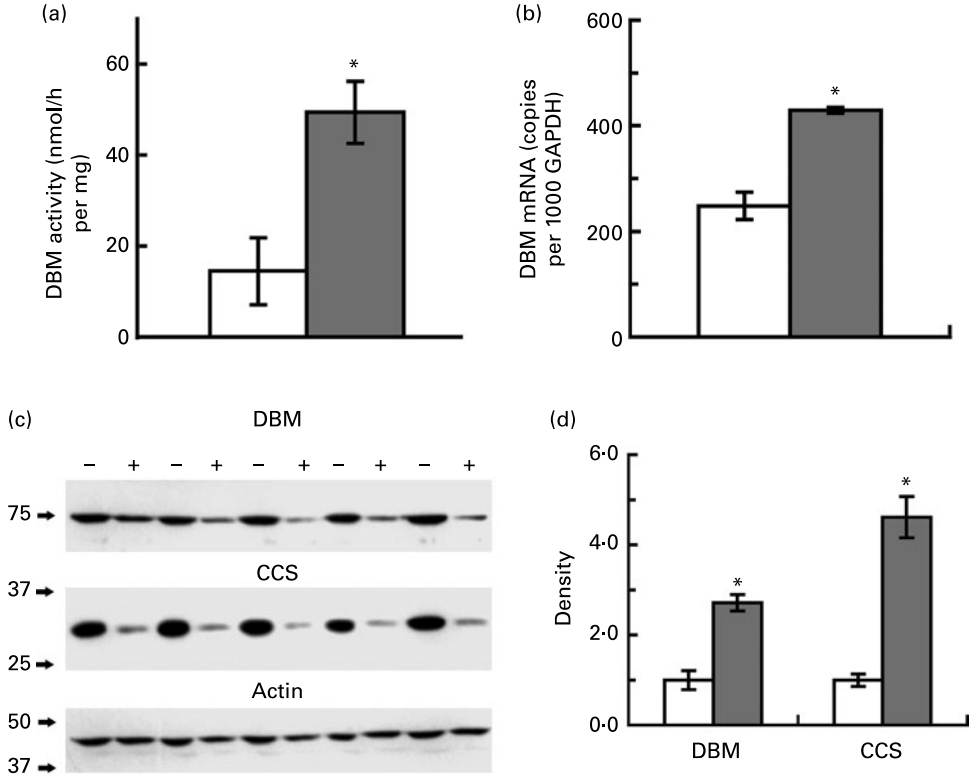

Fig. 2. Adrenal gland (AG) dopamine β-mono-oxygenase (DBM) evaluation. (a) DBM activity of postnatal day 24 male Holtzman rats following perinatal Cu deficiency. (□), Cu-adequate samples; (■), Cu-deficient samples. (b) Copy number of DBM and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined by using quantitative real-time PCR from postnatal day 25 male rats. (c) DBM protein abundance (15 μg) in postnatal day 24 male rats was analysed by Western blot. Cu status was evaluated by the marker Cu chaperone for superoxide dismutase (CCS). Actin was used as a loading control. (d) Densitometry means were determined for Cu-adequate and Cu-deficient AG samples. DBM protein levels in Cu-deficient samples were significantly higher compared with Cu-adequate samples. Values are means (n 5), with standard errors represented by vertical bars. * Mean value was significantly different from that of the Cu-adequate rats ($P<0·05$; Student's t test).

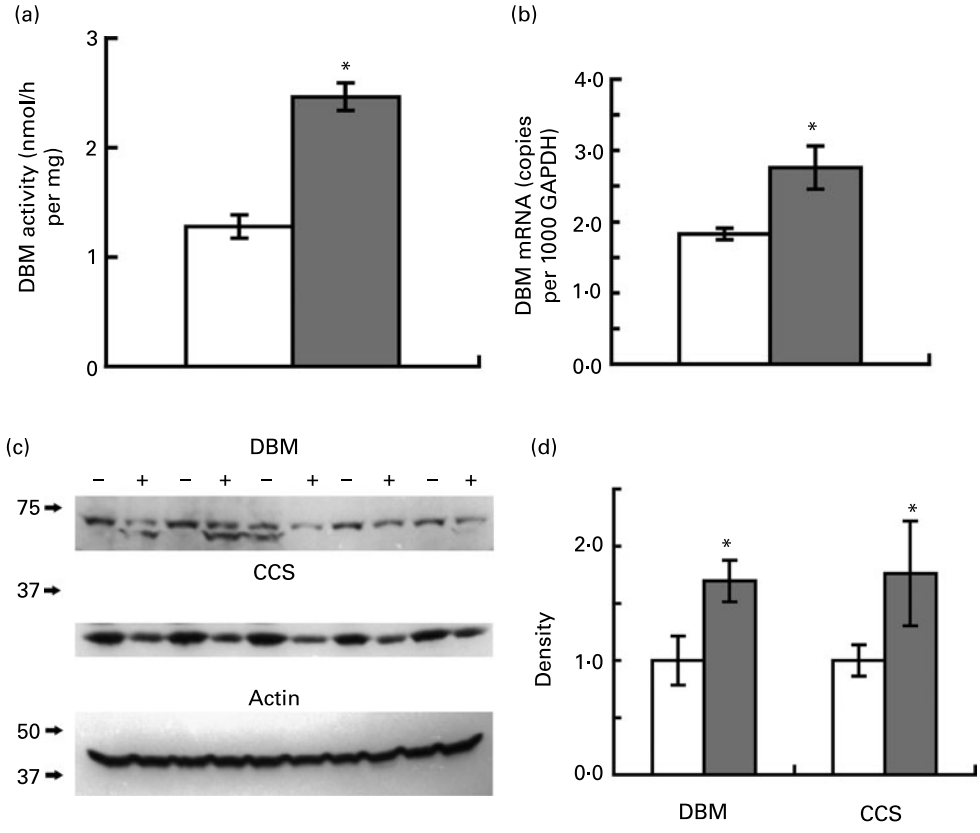

Fig. 3. Medulla oblongata/pons (MO) dopamine β-mono-oxygenase (DBM) evaluation. (a) DBM activity of postnatal day 24 male Holtzman rats following perinatal Cu deficiency. (□), Cu-adequate samples; (■), Cu-deficient samples. (b) Copy number of DBM and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined by using quantitative real-time PCR from postnatal day 25 male rats. (c) DBM protein abundance (75 μg) in postnatal day 26 male rats was analysed by Western blot. Cu status was evaluated by the marker Cu chaperone for superoxide dismutase (CCS). Actin was used as a loading control. (d) Densitometry means were determined for Cu-adequate and Cu-deficient MO samples. DBM protein levels in Cu-deficient samples were significantly higher compared with Cu-adequate samples. Values are means (n 5), with standard errors represented by vertical bars. * Mean value was significantly different from that of the Cu-adequate rats ($P<0·05$; Student's t test).

samples (Fig. 3 (c)). Actin was not changed by treatment (Fig. 3 (c)). The augmentation of DBM in Cu− medulla oblongata/pons tissue was not as high as in adrenal glands. The mean DBM density of Cu− samples was 0·7-fold higher than that of the Cu+ samples (Fig. 3 (d)). The mean CCS density in Cu− medulla oblongata/pons samples displayed a similar 0·8-fold increase.

Vasa deferentia analysis

DBM activity measured in homogenates from vas deferens from postnatal day 25 male rats was not statistically influenced by Cu deficiency (Fig. 4 (a)).

However, surprisingly the number of copies of DBM transcript per 1000 copies of GAPDH transcript was significantly higher in Cu− compared with Cu+ vas deferens (Fig. 4 (b)). The Cu− samples were 2·4-fold higher than the Cu+ samples. The GAPDH copy number was not different between the two groups. Relative mRNA expression of DBM in Cu+ samples in vas deferens was similar to medulla oblongata/pons.

DBM activity in the vas deferens suggested that there was no enhancement in DBM protein abundance, in contrast to adrenal glands and medulla oblongata/pons, but DBM transcript data suggested otherwise. Thus, vas deferens DBM protein abundance of postnatal day 24 male rats was measured by Western immunoblot and, like DBM activity, was not statistically higher in Cu− samples. Vas deferens actin levels were not altered by diet (Fig. 4 (c)). Mean CCS density did reveal a modest and statistically significant 0·4-fold higher abundance in Cu− compared with Cu+ vas deferens extracts consistent with marginal Cu deficiency in vas deferens (Fig. 4 (d)).

Mouse analysis

Mouse adrenal gland and vas deferens DBM protein abundance was analysed to determine if mice respond similarly to rats when subjected to Cu deficiency. Adrenal gland tissue was from postnatal day 27 male mice and vas deferens tissue from postnatal day 28 male mice. The two tissues were evaluated separately. It was apparent that both DBM and CCS protein abundance in adrenal glands was higher in the Cu− samples compared with Cu+ samples (Fig. 5 (a)). Mouse vas deferens DBM protein was detected in Cu− samples but not in Cu+ samples (Fig. 5 (b)). CCS protein was clearly more abundant in Cu− vas deferens samples, similar to

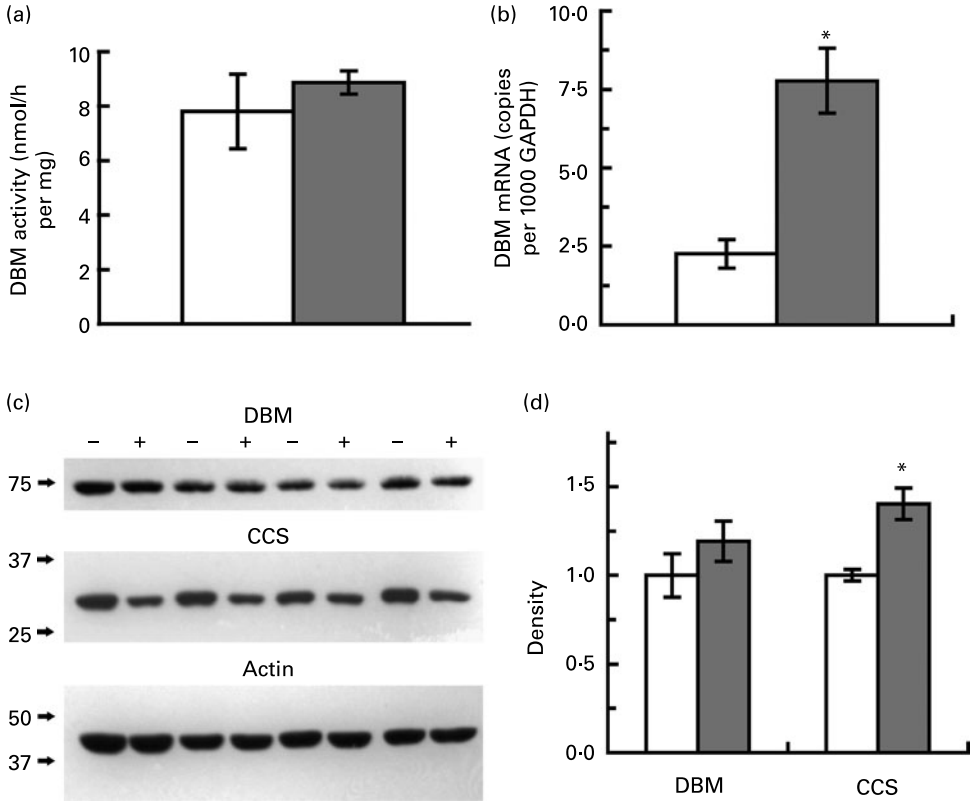

Fig. 4. Vas deferens dopamine β-mono-oxygenase (DBM) evaluation. (a) DBM activity of postnatal day 25 (P25) male Holtzman rats following perinatal Cu deficiency. (□), Cu-adequate samples; (■), Cu-deficient samples. (b) Copy number of DBM and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined by quantitative real-time PCR in P25 male rats. (c) DBM vas deferens protein abundance (15 μg) in postnatal day 24 male rats was analysed by Western blot. Cu status was evaluated by the marker Cu chaperone for superoxide dismutase (CCS). Actin was used as a loading control. (d) Densitometry means were determined for Cu-adequate and Cu-deficient vas deferens samples. DBM protein levels in Cu-deficient samples were not different from those of Cu-adequate samples. Values are means (n 4), with standard errors represented by vertical bars. * Mean value was significantly different from that of the Cu-adequate rats ($P<0·05$; Student's t test).

rat tissue. Ponceau S stain was used as a loading control in mouse tissue because actin data were unreliable in mice. Consistent with immunoblot data, mean adrenal gland DBM activity was higher in Cu− samples (129 (SEM 19) nmol/h per mg) compared with Cu+ samples (76·3 (SEM 7·3) nmol/h per mg) for mouse adrenal glands (n 4; $P<0·05$).

Expression of copper-related genes

Copper-related genes in addition to DBM were studied to determine if the DBM transcription abundance enhancement was unique. Medulla oblongata/pons relative mRNA expression in postnatal day 25 male rats was determined for

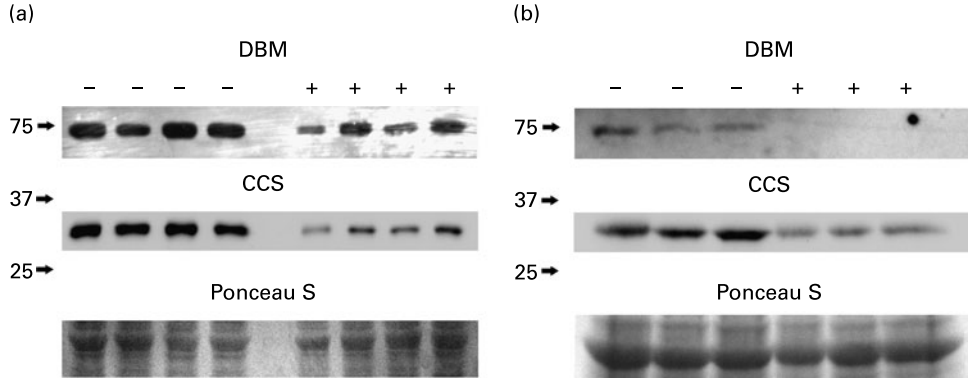

Fig. 5. Evaluation of mouse dopamine β-mono-oxygenase (DBM). (a) DBM protein abundance of adrenal glands of postnatal day 27 male mice following Cu deficiency was analysed by Western blot. Cu chaperone for superoxide dismutase (CCS) was used to confirm Cu deficiency. Ponceau S stain was used as a loading control; 30 μg protein was loaded. Cu-deficient (−) samples had significantly higher abundance of both DBM and CCS protein compared with Cu-adequate (+) samples. (b) DBM and CCS abundance in vas deferens of postnatal day 28 male mice following Cu deficiency was analysed by Western blot. Ponceau S stain was used as a loading control; 50 μg protein was loaded. Cu-deficient (−) samples had significantly higher levels of both DBM and CCS protein compared with Cu-adequate (+) samples.

the following genes: DBM, CCS, cytochrome c oxidase subunit 1 (COX I), cytochrome c oxidase subunit 4 (COX IV), Cu transporter (SLC31A1) (CTR1), peptidylglycine α-amidating mono-oxygenase (PAM) and Cu,Zn superoxide dismutase (*EC* 1.15.1.1) (SOD1) using primer pairs for qRT-PCR (Table 1). Only DBM showed a statistical difference between Cu− and Cu+ samples. Even though protein abundance for the CCS was elevated in Cu deficiency (Fig. 3 (c)), CCS mRNA was not altered by diet. The abundance of DBM mRNA in medulla oblongata/pons was very low compared with cuproenzymes SOD1 or cytochrome c oxidase, and similar to PAM.

Catecholamine analysis

Catecholamines were extracted from postnatal day 26 male rat adrenal glands, medulla oblongata/pons and vas deferens, and from P24 male rat heart. HPLC analyses demonstrated that diet did not have a significant effect on total NA in adrenal glands. However, total DA and DA:NA ratio (100 × DA/NA) were significantly higher in Cu− compared with Cu+ rats (Fig. 6 (a)), a 2-fold increase for both. Cu deficiency also had a significant effect in medulla oblongata/pons. NA concentration was lower by 25 % in Cu− compared with Cu+ tissue and DA concentration and DA : NA ratio was higher by 1·2-fold and 2·2-fold, respectively, in Cu− compared with Cu+ medulla oblongata/pons (Fig. 6 (b)). Cu deficiency also had a significant effect in vas deferens and heart. Total NA in vas deferens was lower by 29 % in Cu− compared with Cu+ rats and DA concentration and DA:NA ratio were elevated by approximately 0·6-fold and 1·1-fold, respectively, in Cu− compared with Cu+ tissue (Fig. 6 (c)). Total NA in heart was lower by 64 % in Cu− compared with Cu+ rats and DA concentration and DA:NA ratio were markedly higher by 15-fold and 48-fold, respectively, in Cu− compared with Cu+ rats (Fig. 6 (d)). Collectively, these catecholamine data suggest that Cu deficiency limits DBM activity *in vivo*.

Correlation of dopamine β-mono-oxygenase mRNA expression v. noradrenaline concentration

To compare DBM mRNA elevation with NA depletion in tissues, heart DBM copy number was first determined by qRT-PCR. There was no statistical difference between DBM copy number in Cu− and Cu+ heart samples when normalised to 1000 copies of GAPDH (Fig. 7 (a)). However, unlike other tissues studied, GAPDH copy number was slightly higher in Cu− than Cu+ heart. Thus, 18S ribosomal RNA abundance was determined and when using an average of both loading controls, GAPDH and 18S, there was a modest increase (0·4-fold) in DBM copy number ($P<0·05$). DBM mRNA abundance of heart was the lowest of all tissues studied, even lower than medulla oblongata/pons (Fig. 3 (b)).

DBM mRNA elevation may be due to NA reduction; therefore, DBM mRNA elevation in four Cu− tissue samples was

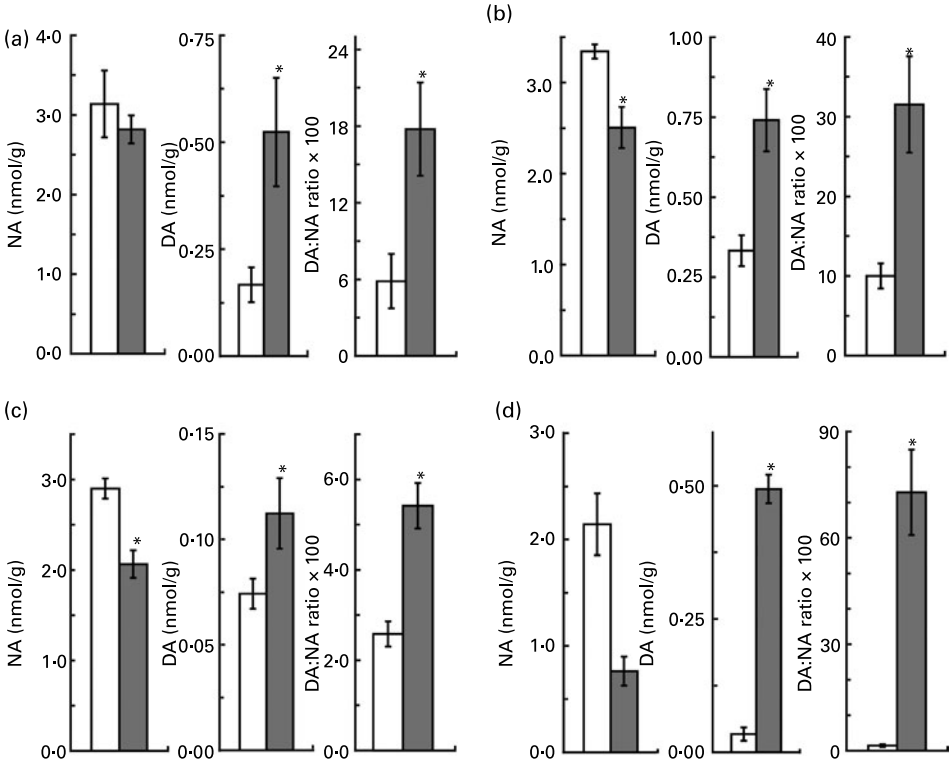

Fig. 6. Measurement of rat catecholamine concentrations by HPLC with electrochemical detection. Noradrenaline (NA) and dopamine (DA) levels were determined following perinatal Cu deficiency in male rat adrenal glands (a), medulla oblongata/pons (b), vasa deferentia (c) and heart (d). For adrenal glands, medulla oblongata/pons and vasa deferentia, rats at postnatal day 26 were sampled. For heart, rats at postnatal day 24 were sampled. (□), Cu-adequate samples; (■), Cu-deficient samples. Values are means (n 3–5), with standard errors represented by vertical bars. * Mean value was significantly different from that of the Cu-adequate rats ($P<0·05$; Student's t test).

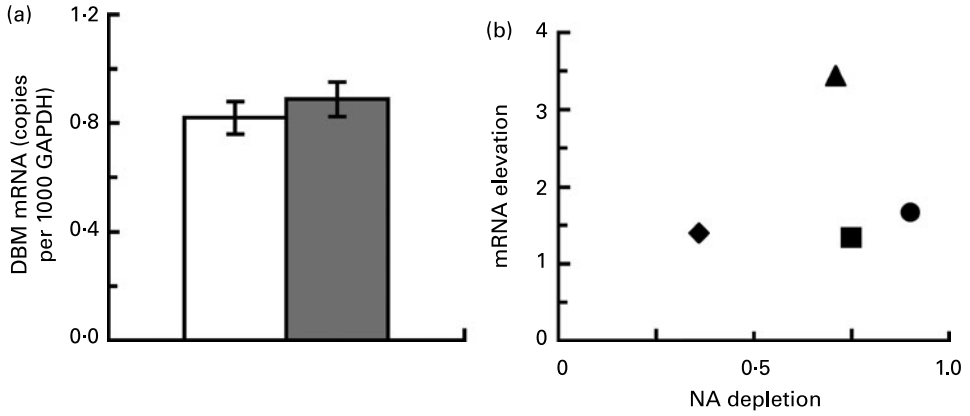

Fig. 7. Abundance of dopamine β-mono-oxygenase (DBM) mRNA in rat heart and relationship of the enhancement of DBM mRNA with noradrenaline (NA) deficit in Cu-deficient tissues. (a) Copy number of DBM and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was determined using quantitative real-time PCR in the heart of postnatal day 26 male rats. DBM copy number/1000 GAPDH means were compared in Cu-adequate (□) and Cu-deficient (■) heart samples. Values are means (n 3), with standard errors represented by vertical bars. No significant differences were detected. (b) DBM mRNA transcriptional response compared with noradrenaline (NA) depletion in Cu-deficient rat tissues. Normalised mRNA changes *v.* NA depletion were plotted for adrenal glands (●), medulla oblongata/pons (■), vasa deferentia (▲) and heart (◆). A correlation coefficient of 0·2 was determined ($P>0·05$).

plotted *v.* NA reduction (Fig. 7 (b)). The correlation coefficient for these four data pairs was calculated to be 0·2, and not statistically significant.

Discussion

The model for perinatal Cu deficiency in the present experiments produced rats that were severely deficient based on growth retardation, cardiac hypertrophy, and highly reduced brain and liver Cu levels. The results from adrenal, medulla oblongata/pons, vas deferens and heart strongly confirm and extend limited previous data that dietary Cu deficiency leads to an augmentation in DBM mRNA levels. Presently no data are available to evaluate DBM mRNA degradation in Cu− rats. Thus, we suggest that there is increased transcription of DBM following Cu deficiency. Past studies of Cu-deficient rat adrenals showed that there was a significant elevation of DBM mRNA evaluated by Northern blots[18]. The same study showed an elevation in DBM mRNA in brain of Cu− female but not male rat pups. It is possible that multiple factors contribute to the up-regulation of DBM mRNA abundance as message levels are transcriptionally regulated by several factors including cAMP, glucocorticoids, bradykinin, nicotine and immobilisation stress[30].

However, reduction of NA levels in Cu− tissues seemed like the most logical candidate for the increased transcription of DBM. Induction of rat adrenal DBM mRNA can be achieved by a treatment with reserpine, a drug that depletes catecholamines[19]. DBM activity and protein levels in the brain of rats were elevated when treated with reserpine, while catecholamines were lower[20]. Consistently lower NA levels in medulla oblongata/pons of Cu-deficient rodents support the hypothesis that depleted NA levels drive the increased DBM mRNA transcription. However, our present results and past work show some inconsistencies in the hypothesis, particularly in adrenal tissue. Studies in older rats subjected to Cu deficiency after weaning found that rat adrenal NA was lower and DBM activity higher following Cu deficiency[18,31]. However, the present studies, as well as others, showed no significant reduction in NA content and yet increased DBM mRNA[18]. Additionally, Cu-deficient cattle showed an adrenal NA reduction but with no change in DBM activity[32]. Rat heart NA levels showed the greatest depletion of all the tissues studied in Cu− samples in the present studies, yet only a modest change in DBM mRNA and only when DBM copy number was normalised to two controls. The reduction in NA content and the DBM mRNA elevation in the four tissues studied in the present experiments were not statistically correlated. Thus, the mechanism for increased DBM mRNA transcription is still not well defined. One possibility is altered glucocorticoids since DBM transcription is augmented by glucocorticoids. This is an ideal candidate because glucocorticoids are elevated in both the adrenal glands and plasma of Cu− rats[33]. However, Cu− mice also have higher DBM protein and activity, and perhaps mRNA levels. However, Cu− mice do not have higher glucocorticoids in plasma[34]. Higher DBM mRNA content in Cu− tissues could be due to increased transcript stability rather than enhanced synthesis. Future research is needed to fully understand the regulation of DBM following Cu deficiency.

However, our data clearly indicate that increased DBM mRNA abundance is associated with an increase in DBM protein. The present data confirm that in young Cu-deficient rats increased adrenal DBM mRNA and increased DBM protein are evident[17,18]. Novel data report that DBM protein abundance in the medulla oblongata/pons by Western blot is higher in DBM protein in Cu− young male rats. Mouse adrenal and vas deferens samples also demonstrated higher DBM protein, demonstrating that mice responded in a similar manner to rats to Cu deprivation.

Higher DBM protein in Cu− animals is consistent with higher *in vitro* DBM activity and confirms similar changes in DBM activity and protein reported previously for adrenal glands from young rats[17]. The present results also show that higher DBM activity *in vitro* and higher DBM protein were evident in both adrenal and medulla/pons tissue of Cu− rats. The magnitude of change in both activity and protein are very similar (adrenal activity 2·4-fold *v.* protein 1·7-fold change and medulla/pons activity 0·9-fold *v.* protein 0·7-fold change). Though there was no significant difference

in Cu− rats compared with Cu+ rats for vas deferens for either DBM activity or protein levels, a trend of change in both was similar (activity 0·2-fold *v.* protein 0·14-fold change). Note that a very modest change for CCS in Cu− vas deferens was detected, suggesting a more marginal Cu deficiency in this tissue. There was an excellent correlation in Cu− rat tissues between augmented DBM protein and DBM *in vitro* activity in Cu− medulla/pons, adrenal glands and vas deferens of 0·98 ($P<0·05$). This would suggest that the higher DBM activity observed in tissues from Cu− mammals is due to a higher abundance of DBM protein rather than changes in endogenous inhibitors or subtle kinetic differences observed previously[16,31].

Despite higher DBM activity *in vitro*, catecholamine results suggest that DBM activity *in vivo* is lower in Cu− animals. As shown in the present study, NA is significantly lower in medulla/pons, vas deferens and heart of Cu− rats. DA levels were significantly higher in all four of the tissues studied in Cu− rats. The DA:NA ratio was significantly higher; approximately 2-fold greater in adrenals, medulla and vas deferens in Cu− rats compared with their controls. The ratio in heart was even higher, showing a 48-fold increase in Cu− rats. This is totally consistent with radiotracer studies done in Cu− rat heart suggesting that DBM activity *in vivo* was impaired by Cu deficiency[9]. Catecholamine changes in mouse heart and medulla oblongata/pons also respond in a similar manner to Cu deficiency[21]. Present and past data support the hypothesis that there is higher apo-DBM in tissues from Cu-deficient mammals but function is limited by lower Cu availability. Data in the present studies were obtained from a severe restriction in dietary Cu. Thus, the impact of more marginal Cu limitation to DBM function is unknown. Our work and that of others have indicated that DBM limitation can occur in a variety of mammals and that the response is both species and organ specific[35–37].

The DA:NA ratio in serum can provide valuable information to the clinical community. Clinical and pathological features of Menkes' disease often reflect decreased activities of cuproenzymes, including DBM and others[38]. A significantly raised DA:NA ratio is a promising test for neonatal infants suspected of Menkes' disease[39]. Additionally, a higher DA:NA ratio was shown in 100 % of the first six cases of DBM deficiency in humans[40]. Thus, the DA:NA ratio is a useful diagnostic tool for DBM limitation.

DBM is an important cuproenzyme. The low copy number of DBM mRNA and immunoblot data suggest that it is not a very abundant protein in the central nervous system. However, despite its low abundance, its function clearly is vital to the livelihood of animals, as demonstrated by the DBM knockout mouse studies[8]. In humans, patients that have DBM deficiency have cardiovascular disorders and severe orthostatic hypotension. Regardless of the mechanisms for controlling DBM levels our data and others are congruent that DBM function *in vivo* requires adequate Cu; thus DBM is a key cuproenzyme in mammals.

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