Effect of dietary copper deficiency on the distribution of dopamine and norepinephrine in mice and rats

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Dietary copper deficiency was produced in Swiss albino mice and Sprague Dawley rats to determine the organ specificity of alterations in norepinephrine (NE) and dopamine (DA) concentrations and the relationship with organ copper levels. A 5-week dietary treatment was used, which started 1 week after birth for mice, initially via dams, and 3 weeks after birth for rats. Mice offspring (6 weeks of age) and rats (8 weeks of age) maintained on a copper-deficient (−Cu) treatment were compared with copper-adequate (+Cu) controls. Compared with +Cu animals, −Cu mice and rats were anemic and had low (< 1% of +Cu) ceruloplasmin activities but normal body weights. The −Cu mice had organ copper concentrations ranging between 30% and 65% of +Cu values for eight organs studied, with the thymus being the least depleted. For −Cu rats, the range was 15% to 65%. Significant reductions in NE concentration were observed in the heart, pancreas, and spleen of −Cu mice. Elevated DA levels were observed in all organs except the brain. For −Cu rats, the NE level was lower in the heart and the DA level was higher in both the heart and spleen compared with +Cu rats. Dopamine elevation in the heart and spleen for both −Cu mice and rats was four- and fivefold higher, respectively. Adrenal catecholamine levels were only slightly changed by copper deficiency in mice or rats. Urinary levels of both NE and DA were higher in −Cu rats and mice. Plasma and heart tyrosine levels were not altered in −Cu mice. Elevated DA in −Cu rodents may be due to limiting dopamine-β-monooxygenase. Higher urinary NE and lower organ NE may be due to a combination of decreased synthesis and enhanced turnover. The magnitude of decreased organ copper was not predictive of altered catecholamine pool size.

Keywords: Copper deficiency; mice; rats; norepinephrine; dopamine

Introduction

Copper is one of several essential trace elements that has an important role in catecholamine metabolism.[1] The cuproenzyme, dopamine-β-monooxygenase (DBM) (EC 1.14.17.1), catalyzes the conversion of dopamine (DA) to norepinephrine (NE) in noradrenergic neurons and in the adrenal medulla. Normally, DBM activity is not rate-limiting in NE biosynthesis, as this pathway is thought to be regulated by the activity of tyrosine-3-monooxygenase and the availability of tyrosine.[2] However, more than 20 years ago, it was demonstrated that DBM activity could be rate-limiting in NE synthesis.[3] Hearts from copper-deficient (−Cu) rats converted less [^{14}C]dopamine to [^{14}C]norepinephrine compared with copper-adequate (+Cu) controls. However, several attempts to directly assay DBM activity in vitro following copper deficiency have shown that DBM activity is elevated rather than reduced, leaving an unresolved puzzle.[4-6]

If DBM activity is functionally rate-limiting in vivo, one would predict that steady-state levels of catecholamines would be altered, namely, depression of NE and elevation of DA. Brains of suckling −Cu rats do have lower NE levels compared with controls.[7] Brains of −Cu lambs also exhibit this alteration.[8] However, DA alteration has been somewhat of an enigma. O'Dell and colleagues have shown that brains of −Cu lambs[8] and rats[9] have lower DA levels compared with

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controls. Repletion of rats with copper returned NE levels to normal but did not reverse the DA deficit; for DA, this suggests loss of cells rather than reduced DBM activity as an explanation.[10] There may be some other factor in addition to the lack of copper that explains the DA effect because not all −Cu rats have altered DA.[11] In fact, other investigators have not observed alterations in whole brain DA in −Cu rats or mice.[6]

In the peripheral nervous system, lower NE concentrations have been reported in the hearts of −Cu rats[12,13] and in the duodenal and colonic muscle of −Cu steers.[14] In one study, the levels of DA in the hearts of −Cu rats were equivalent to pair-fed controls, but slightly higher than ad libitum-fed controls.[13] In a preliminary study with C57BL mice, −Cu animals had elevated DA in both the heart and spleen, but no changes in the brain.[15] It is apparent that some differences exist in the current literature regarding the effects of copper deficiency on catecholamine levels in rodents.

It was the purpose of the current study to examine steady-state levels of NE and DA in a number of organs from both mice and rats following copper deficiency. A second purpose was to determine if putative changes correlate with severity of deficiency as judged by organ copper levels.

Methods and Materials

Animal care and diets

Swiss albino mice, Hsd:(ND/4) (S)BR, purchased commercially (Harlan Sprague Dawley, Indianapolis, IN, USA), were mated to establish a breeding colony. Two days following parturition, the litter size was adjusted to eight pups. The same supplier provided male weanling Sprague Dawley rats.

Four days after parturition, the mouse dams were divided into two dietary treatments, −Cu and +Cu, which consisted of feeding a −Cu purified diet (Teklad Laboratories, Madison, WI, USA) and either low-copper drinking water or copper-supplemented drinking water, respectively. The purified diet was similar to the AIN-76A diet,[16,17] and contained the following major components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; and AIN-76A vitamin mix, 10. Cupric carbonate was omitted from the AIN-76 mineral mix. By analysis, the purified diet contained 0.43 mg copper/kg and 50 mg iron/kg. Offspring and dams on the −Cu treatment drank deionized water containing 0.2 ng copper/l by analysis, whereas +Cu treatment groups drank water than contained 20 mg copper/l by adding copper to the drinking water as $CuSO_4$. Diet and drinking water were available ad libitum.

Male mice were weaned at 3 weeks of age, placed in stainless steel cages (four mice per cage), and were maintained on the same treatment as their respective dams for an additional 3 weeks. Male weanling rats 3 weeks of age were randomly assigned to the +Cu or −Cu treatments, were housed individually in stainless steel cages, and were kept on treatment until 8 weeks of age. All animals were maintained at 24°C with 55% relative humidity on a 12-hour light cycle (7:00 AM–7:00 PM).

Sample collection

Urine was collected over a 24- or 48-hour period into clean plastic tubes that contained 0.1 ml of 6 N HCl. Mice and rats were allowed to adjust to the metabolism cages (Model 650-0311, Nalge Co., Rochester, NY, USA) for 24 hours prior to sample collection. Urine was filtered through screening columns (11-387-50, Fisher Scientific, Pittsburgh, PA, USA) and stored at −70°C for short intervals.

Blood samples were drawn into heparinized microhematocrit tubes under light ether anesthesia from the retroorbital plexus for mice, or were obtained by cardiac puncture while under halothane anesthesia for rats.

Mice (6-week-old males) and rats (8-week-old males) were killed by decapitation and a variety of organs were removed, weighed, and processed for biochemical analysis or frozen in liquid nitrogen. Organs for catecholamine analysis were frozen at −70°C for no longer than 1 week prior to analysis.

Biochemical analyses

Urinary creatinine was determined by analysis of 5-μl (mice) or 2-μl (rat) samples using minor modifications of an alkaline-picric acid procedure (kit no. 555A, Sigma Chemical, St. Louis, MO, USA). Volumes used were 20% of those suggested. Absorbance was determined at 500 nm (Model DU-8 Spectrophotometer, Beckman Instruments, Inc., Fullerton, CA, USA). Hemoglobin was determined spectrophotometrically as metcyanhemoglobin.[18] Plasma was obtained by centrifugation, and the activity of the cuproprotein ceruloplasmin was measured by following oxidation of *O*-dianisidine.[18] Plasma and selected heart samples were also analyzed for tyrosine levels. Plasma and heart were mixed with cold 0.6 N trichloroacetic acid, and an aliquot of the supernate was used to determine tyrosine fluorometrically.[19]

Organs (or pools of organs for some mouse samples), livers, and 1-g portions of diets were wet-digested with 4 ml of concentrated HNO_3 (AR select grade, Mallinckrodt, St. Louis, MO, USA), and the residue was brought to 4.0 ml with 0.1 N HNO_3. Samples were then analyzed for total copper and iron by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT, USA). The method was checked with a certified standard, U.S. National Bureau of Standards 1577 bovine liver (Gaithersburg, MD, USA). The mean copper and iron values obtained were 96% and 98%, respectively, of those certified.

Catecholamine analysis

Urine was processed for high-pressure liquid chromatography (HPLC) using a two-step procedure

as described in detail elsewhere.[20] Step one consisted of ion-exchange chromatography and step two, adsorption to alumina. Adrenal glands were homogenized (Tissumizer, Tekmar Co., Cincinnati, OH, USA) in cold 0.05 N $HClO_4$, and a 50-μl aliquot of supernate was used for HPLC analysis. Organs were processed for catecholamine analysis using a protocol described in detail previously.[20] Binding to and elution from alumina were carried out at room temperature; samples were kept on ice until fractionated by HPLC.

The extracted catecholamines were fractionated by reverse-phase ion-pair HPLC with electrochemical detection.[20] The mobile phase was composed of an aqueous buffer (0.1 M potassium phosphate, 0.1 mM EDTA, 0.15 mM sodium octylsulfonate, pH 3.0), 95%, and methanol (HPLC grade, Fisher Scientific), 5%. Chromatography was carried out at room temperature, and the eluant from the column was heated to 32°C prior to detection. The system consisted of a dual-piston pump (Kratos Spectroflow 400, Applied Biosystems, Foster City, CA, USA), an amperometric detector (LC-4B), temperature controller (LC-22A), and flow cell housing (LC-17A) from Bioanalytical Systems, Inc. (West Lafayette, IN, USA) equipped with a Rheodyne injector (Model 7125, Rheodyne, Inc., Cotati, CA, USA). The compounds were fractionated on a 4.6 × 250 mm analytical column (Ultrasphere ODS 5 μM, Beckman Instruments, Inc.) preceded by a 3.2 × 15 mm cartridge guard column (Aquapore ODS 7 μM, Brownlee Labs, Applied Biosystems, Foster City, CA, USA). Flow rate was maintained at 1.2 ml/min. The glassy-carbon electrode was set at 0.7 V versus Ag/AgCl. Output was recorded and peak areas were integrated (C-R3A, Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

Statistical analysis

Mean comparisons between the two treatment groups were made using the Student's t test, $\alpha = 0.01$. Regression analysis was performed to determine the best linear fit of data for creatinine and tyrosine standards using a Macintosh personal computer and statistical software (Statview 512+, Brain Power, Calabasas, CA, USA).

Results

The mice and rats used in these experiments grew well on both the −Cu and +Cu treatments, and body weights were not altered (*Table 1*). The −Cu treatment did, however, result in anemia and low plasma ceruloplasmin activities.

Eight different tissues and organs were analyzed from the 6-week-old male mice. Compared with +Cu mice, the weights of the hearts and spleens were higher in −Cu mice and the weights of the thymuses were lower (*Table 2*). There were no significant differences in the weights of the brain, cerebellum, kidney, liver, or pancreas between +Cu and −Cu mice. The concentration of copper (μg/g wet weight) was lower for all eight mouse tissues examined from −Cu mice compared with +Cu mice (*Table 2*). The organ least depleted was the thymus, in which the mean −Cu value was 65% of the +Cu value. The organs most depleted were the heart, cerebellum, and spleen, in which the mean values for −Cu mice were 29%, 30%, and 30% of the +Cu mean values, respectively.

The NE and DA content of organs obtained from mice with similar characteristics to those used for metal analysis (degree of anemia and liver copper levels) were determined by HPLC (*Table 3*). Despite the copper deficit in the organs examined, the concentrations of NE were lower only for the heart, pancreas, and spleen in −Cu mice compared with +Cu mice. In contrast, there was a significant increase in organ DA level in −Cu mice in all organs but the brain (*Table 3*). This ranged from an approximate doubling for the cerebellum, kidney, pancreas, and thymus to more than fourfold for the heart and fivefold for the spleen. However, the quantitative drop in organ NE (nmol/g) was larger than the corresponding rise in DA.

The relative degree of copper deficiency in an organ was determined by calculating the percentage of the comparative +Cu value for organ copper level. This value was plotted in a scattergram versus the corresponding decrease in organ NE (% +Cu value) and increase in organ DA (% +Cu value) (*Figure 1*). For the seven organs studied, there was no apparent linear relationship between these variables ($P > 0.01$). That is, an organ low in copper did not necessarily have a low NE and a high DA level.

Male rats were sampled when at 8 weeks of age for changes in organ weight and tissue copper levels (*Table 4*). In comparison with +Cu rats, the −Cu rats had an increased heart weight and a lower thymus weight. The copper concentration of the six organs sampled was lower in the −Cu rats compared with +Cu rats. The organ least depleted of copper was the

Table 1 Characteristics of copper-adequate and -deficient mice and rats[a]

Parameter	Mice		Rats	
	+Cu	−Cu	+Cu	−Cu
Age (wk)	6	6	8	8
Body weight (g)	20.3 ± 0.33	19.7 ± 1.0	284 ± 11.4	269 ± 7.92
Hemoglobin (g/100 ml)	14.9 ± 0.26	6.53 ± 0.84[b]	13.6 ± 0.84	10.2 ± 0.36[b]
Ceruloplasmin (U/l)	34.9 ± 0.84	0[b]	122 ± 3.70	0.76 ± 0.14[b]

[a] Values are means ± SEM for nine mice or six rats of each treatment; hemoglobin levels and ceruloplasmin activity were determined spectrophotometrically; mean values were compared by Student's t test
[b] $P < 0.01$

Table 2 Organ weights and copper levels of 6-week-old male Swiss albino mice[a]

Organ	Weight (mg)[b]		Copper (μg/g)	
	+Cu	−Cu	+Cu	−Cu
Brain	430 ± 11.2 (8)	403 ± 8.76 (8)	4.11 ± 0.07 (5)	1.52 ± 0.15 (5)[c]
Cerebellum	58.0 ± 2.37 (8)	56.9 ± 3.18 (8)	4.46 ± 0.14 (8)	1.32 ± 0.51 (8)[c]
Heart	115 ± 2.95 (8)	151 ± 5.81 (8)[c]	6.79 ± 0.20 (4)	1.94 ± 0.28 (4)[c]
Kidney	174 ± 13.5 (4)	158 ± 6.75 (4)	4.92 ± 0.08 (9)	2.47 ± 0.06 (9)[c]
Liver	1.09 ± 0.07 (9)	1.19 ± 0.05 (9)	4.79 ± 0.17 (9)	1.69 ± 0.08 (9)[c]
Pancreas	100 ± 4.11 (4)	89.0 ± 9.39 (4)	1.48 ± 0.05 (6)	0.605 ± 0.033 (4)[c]
Spleen	63.2 ± 2.19 (8)	96.2 ± 11.34 (8)[c]	0.922 ± 0.023 (5)	0.277 ± 0.029 (7)[c]
Thymus	61.1 ± 2.66 (6)	35.5 ± 4.17 (6) [c]	0.820 ± 0.034 (6)	0.532 ± 0.045 (4)[c]

[a] Values for organ weights are means ± SEM for the number in parentheses of individual organs; values for copper levels are means ± SEM for the number in parentheses of individual organs (brain, cerebellum, heart, kidney, and liver) or number of respective pools (pancreas, spleen, and thymus) consisting of at least three organs per pool; copper values are expressed as μg/g wet weight; means were compared by Student's *t* test
[b] Liver weight in grams
[c] $P < 0.01$

Table 3 Organ catecholamine levels of 6-week-old male Swiss albino mice[a]

Organ	NE (ng/g)		DA (ng/g)	
	+Cu	−Cu	+Cu	−Cu
Brain	542 ± 17.8 (8)	526 ± 34.1 (8)	1100 ± 30.6 (8)	1109 ± 68.3 (8)
Cerebellum	344 ± 43.4 (7)	338 ± 22.2 (7)	14.6 ± 2.96 (7)	33.6 ± 3.56 (7)[b]
Heart	908 ± 78.1 (8)	240 ± 62.4 (8)[b]	30.2 ± 4.21 (8)	127 ± 10.4 (8)[b]
Kidney	371 ± 23.5 (4)	335 ± 14.5 (4)	36.6 ± 2.71 (4)	76.0 ± 5.58 (4)[b]
Pancreas	294 ± 14.5 (4)	153 ± 24.9 (4)[b]	18.9 ± 1.37 (4)	40.7 ± 1.94 (4)[b]
Spleen	517 ± 44.6 (8)	233 ± 31.4 (8)[b]	37.0 ± 3.15 (8)	196 ± 57.0 (8)[b]
Thymus	102 ± 10.4 (6)	129 ± 20.6 (6)	10.4 ± 0.94 (6)	29.7 ± 3.44 (6)[b]

[a] Values are means ± SEM for the number, in parentheses, of individual organs; catecholamine levels were determined by HPLC and are expressed on a fresh weight basis; means were compared by Student's *t* test
[b] $P < 0.01$

Table 4 Organ weight and copper levels of 8-week-old Sprague Dawley rats[a]

Organ	Weight (g)		Copper (μg/g)	
	+Cu	−Cu	+Cu	−Cu
Brain	1.96 ± 0.11	1.96 ± 0.09	2.58 ± 0.06	1.66 ± 0.06[b]
Heart	0.93 ± 0.02	1.19 ± 0.05[b]	5.05 ± 0.10	1.00 ± 0.10[b]
Kidney	2.41 ± 0.08	2.28 ± 0.05	5.57 ± 0.18	2.86 ± 0.10[b]
Liver	14.4 ± 0.66	14.2 ± 0.57	4.18 ± 0.15	0.63 ± 0.05[b]
Spleen	0.62 ± 0.03	0.57 ± 0.02	1.28 ± 0.07	0.19 ± 0.02[b]
Thymus	0.66 ± 0.03	0.51 ± 0.02[b]	0.81 ± 0.03	0.32 ± 0.03[b]

[a] Values are means ± SEM of six rats from each group; means were compared by Student's *t* test
[b] $P < 0.01$

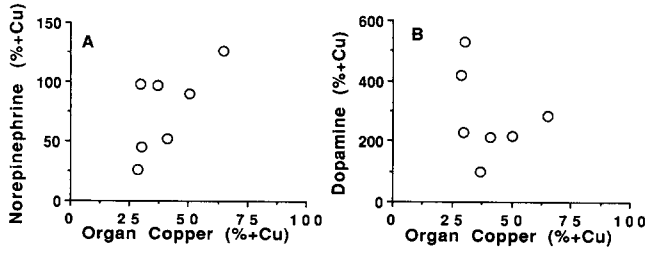

Figure 1 Relationship between copper status in seven organs (% +Cu organ copper levels for −Cu mice) and depression of NE levels (A) or elevation of DA levels (B)

brain, in which the mean −Cu value was 64% of the +Cu value. The spleen, liver, and heart were the most depleted, as the −Cu values were 15%, 15%, and 20%, respectively, of the +Cu values (*Table 4*).

Catecholamine analysis was performed on the brain, heart, and spleen of rats from a sample of the population. Only for the heart was NE lower in −Cu rats (*Table 5*). There was a significant increase for both the heart (4.5-fold) and spleen (5.9-fold) in DA levels in −Cu rats compared with +Cu rats. Brain NE and DA were not altered in −Cu rats.

The content of NE and DA in adrenal glands and urine was also determined for both mice and rats (*Table 6*). For the adrenal gland, there was a modest decrease in NE in the −Cu mice, but not in the −Cu rats. In other experiments, no change in adrenal NE content in −Cu mice has been observed (data not shown). No differences in adrenal epinephrine levels were noted between −Cu and +Cu mice or rats. Dopamine was elevated in the adrenals of −Cu rats but not −Cu mice.

The urinary output of both NE and DA was higher in −Cu compared with +Cu mice and rats (*Table 6*). This has been a consistent finding for −Cu mice but not for −Cu rats (data not shown). For mice, the urinary output of epinephrine was not altered by diet.

To determine whether copper deficiency had al-

Table 5 Organ catecholamine levels of 8-week-old Sprague Dawley rats[a]

Organ	NE (ng/g)		DA (ng/g)	
	+Cu	−Cu	+Cu	−Cu
Brain	397 ± 15.5	364 ± 4.30	1190 ± 27.8	1180 ± 15.5
Heart	966 ± 26.7	579 ± 35.2[b]	22.5 ± 1.27	102 ± 12.9[b]
Spleen	1330 ± 82.3	1053 ± 129	37.2 ± 3.78	220 ± 30.0[b]

[a] Values are means ± SEM for six rats from each treatment group; NE and DA were determined by HPLC; means were compared by Student's *t* test
[b] $P < 0.01$

Table 6 Urinary and adrenal catecholamine levels of mice and rats[a]

	Mice		Rats	
	+Cu	−Cu	+Cu	−Cu
Adrenal (μg)				
NE	2.82 ± 0.16 (6)	1.94 ± 0.13 (6)[b]	8.78 ± 0.67 (5)	7.19 ± 0.71 (6)
EP	7.50 ± 0.54 (6)	6.78 ± 0.31 (6)	20.5 ± 1.1 (5)	20.4 ± 1.0 (6)
DA	0.106 ± 0.011 (6)	0.101 ± 0.012 (6)	0.29 ± 0.04 (5)	1.60 ± 0.30 (6)[b]
Urinary (ng/mg creatinine)				
NE	1093 ± 195 (4)	2076 ± 57.6 (4)[b]	123 ± 16 (6)	280 ± 23 (6)[b]
EP	198 ± 32.0 (4)	151 ± 28.6 (4)	ND	ND
DA	981 ± 140 (4)	1675 ± 146 (4)[b]	185 ± 20 (6)	448 ± 22 (6)[b]

[a] Values are means ± SEM for the number of 6-week-old male Swiss albino mice or 8-week-old male Sprague Dawley rats; mice and rats were kept on their respective treatments for 5 weeks preceding analyses; means were compared by Student's *t* test
[b] $P < 0.01$
Abbreviation: ND, not determined

tered substrate availability for catecholamine synthesis, a limited number of tyrosine analyses were conducted. The fasting plasma tyrosine levels of six +Cu mice was determined to be 0.121 ± 0.006 (SEM) μmol/ml and did not differ from the value for seven −Cu mice of 0.107 ± 0.007. The heart (an organ with major changes in catecholamine levels) was analyzed from the same mice. The heart value for +Cu mice was 0.602 ± 0.009 μmol/g and did not differ from the −Cu value of 0.591 ± 0.011.

Discussion

Swiss albino mice subjected to limiting copper during perinatal development developed signs consistent with moderate to severe copper deficiency although growth was not altered. The decrement in organ copper levels was variable, suggesting a more severe functional deficiency in some organs. Even though copper levels were lower in all organs examined, there was no evidence of functional deficiency in some tissues. In the central nervous system, for example, the brains from −Cu mice had no change in NE or DA levels, although the −Cu brain copper level was 37% of the +Cu value. In a previous study with C57BL mice treated in a similar manner, there was a significant drop in NE level in the brain.[15,21] Perhaps there is a genetic component that influences alterations in brain catecholamines in copper deficiency, since a genetic factor was previously suggested to explain the alteration in brain DA in copper-deficient rats.[11] Even though analysis of whole brain displayed no alteration in NE or DA levels, the current studies do suggest that the cerebella from −Cu mice exhibit reduced DBM activity since DA levels were doubled. The −Cu Sprague Dawley rats used in the present studies displayed signs of copper deficiency, including a 36% decrease in brain copper level. However, neither brain NE nor DA levels were altered, suggesting that postweanling deficiency does not alter brain NE metabolism in contrast to deficiency imposed during perinatal development.[6,7,9-11]

Alterations to catecholamine levels were also variable in the peripheral nervous system. In −Cu mice, for example, NE was lower only in the heart, pancreas, and spleen, and only in the heart in −Cu rats. However, a much more striking result was the consistent observation that copper deficiency was associated with an elevation in DA levels. This is consistent with a functional limitation of DBM activity in copper deficiency.

The changes in DA and NE in organs are likely the result of limiting copper for maximum DBM activity. Tyrosine availability does not appear to be a criterion for differences in catecholamine levels in −Cu mice. The observation that urinary NE is higher in −Cu mice and rats suggests that copper deficiency might be accompanied by enhanced turnover. A recent study

supports this observation.[20] However, the elevated urinary DA and inconsistent rat urinary data imply that further work should be done on this issue.

The adrenal gland NE and epinephrine pools were not greatly altered by copper deficiency in the present studies. Hesketh found lower adrenal NE but normal epinephrine levels in his studies of copper-deficient rats.[5] The apparent difference between that study and the present rat data is not readily explained.

It is clear from these experiments and others that copper deficiency alters the pools of catecholamines in both the central and peripheral nervous systems. The most reasonable explanation for this is impaired synthesis due to limiting DBM activity. It is less clear whether these changes in pool size are associated with any pathophysiology, such as cardiac hypertrophy or altered immune function.

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