Dietary Copper Deficiency Alters Protein Levels of Rat Dopamine β-Monooxygenase and Tyrosine Monooxygenase

JOSEPH R. PROHASKA[1] AND BRUCE BROKATE

Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, Minnesota 55812

Perinatal copper (Cu) deficiency was studied by offering pregnant Sprague Dawley rats a basal diet low in copper, 0.44 mg/kg, and drinking water containing 0 (-Cu) or 20 (+Cu) mg Cu/L as $CuSO_4$ starting at day 7 of gestation and continuing throughout lactation. To investigate dopamine-β-monooxygenase (DBM) and tyrosine monooxygenase (TM) in adrenal gland and brain, offspring were weaned at Day 21 to treatments of their respective dams for 9 days. Offspring, 30 days old, of Cu-deficient (-Cu) dams were smaller, anemic, and had biochemical features characteristic of severe Cu deficiency. Adrenal DBM enzyme activity of 30-day-old -Cu rats was 40% higher than Cu-adequate (+Cu) rats and DBM protein levels, estimated by Western immunoblot, were 45% higher. Adrenal DBM mRNA levels of -Cu rats were 108% higher than +Cu rats. Adrenal TM protein levels of -Cu rats were 39% higher than +Cu rats. Hypothalamus DBM activity was significantly higher in -Cu than +Cu rats but no reproducible changes in DBM or TM protein levels could be detected by Western immunoblots. Diet history did not impact adrenal gland or hypothalamus levels of actin as detected on reblotted membranes. However, activity of the cuproenzyme Cu,Zn-superoxide dismutase was 50% lower and 30% lower, respectively, in extracts from rat adrenal gland and hypothalamus of -Cu than +Cu rats, indicating altered Cu status in the tissues studied. These data suggest that Cu deficiency is associated with increased formation of DBM and TM protein levels in adrenal gland. Further research will be required to determine the chemical signal responsible for this induction and if DBM or TM protein levels change in other tissues. [Exp Biol Med Vol. 226(3):199–207, 2001]

Key words: rat; dopamine-β-monooxygenase; tyrosine monooxygenase; copper-deficient

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[1] To whom correspondence should be addressed at Department of Biochemistry and Molecular Biology, University of Minnesota—Duluth, 10 University Drive, Duluth, MN 55812. E-mail: jprohask@d.umn.edu

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Copper (Cu) is an essential element for cells in the biological kingdom. Homeostasis of Cu is essential since deficient or excessive cellular Cu both lead to pathological events. The essential nature of Cu is due to its cofactor role at the active site of a number of enzymes (1). When limitation in Cu restricts the activity of cuproenzymes altered biochemical and physiological phenotypes are observed (2). For example, hypopigmentation is observed due to decreased melanin formation because tyrosinase activity is lowered. Connective tissue abnormalities are observed because of decreased cross-linking of collagen and elastin due to reduced lysyl oxidase. Hepatic iron overload occurs when ferroxidase (ceruloplasmin) activity is lower. For other cuproenzymes the connection between lower enzyme activity and altered phenotype remains to be established.

Dopamine-β-monooxygenase (DBM) is a cuproenzyme that catalyzes the final step in the biosynthesis of norepinephrine (NE) by hydroxylating dopamine (DA) in an ascorbate- and oxygen-dependent reaction (3). DBM is located in adrenal medulla, sympathetic neurons, and noradrenergic and adrenergic neurons of the brain. DBM is essential for embryonic development of the mouse as demonstrated in recent studies in which the DBM gene was ablated (4). In humans the lack of DBM results in severe hypotension (5). It has not been clearly established if attenuation of DBM activity that occurs when Cu is limiting results in abnormal physiology. In fact the Cu story regarding DBM and catecholamines is quite puzzling.

Radioisotopic studies in rats indicated that dietary Cu deficiency decreased the conversion of cardiac DA to NE (6). Indeed, the steady-state NE concentration is lower in hearts from Cu deficient rats compared to controls (7).

Later it was shown that DA is elevated in hearts of Cu deficient rats supporting the hypothesis that DBM is altered by Cu deficiency in sympathetic nerves (8).

In the central nervous system the original Cu-DBM research is due in large part to the seminal work of Hunt and Johnson who studied mottled mice (9). Mutations at the mottled locus in mice are homologous with mutations in humans with Menkes disease. Brains of mutant mice con-

verted less [^{3}H]tyrosine to NE and more [^{3}H]tyrosine to DA than control litter mates. Hunt and Johnson concluded that the former observation was due to decreased DBM and the latter to enhanced tyrosine monooxygenase (TM) activity, which they directly confirmed. They also reported lower steady-state NE levels in brain of the mutant mice compared to litter mates (9). Two years later Hunt reported that Cu was lower in brains of the mutant mice and the Cu-DBM connection in brain was formulated (10). Independently, it was reported that compared to controls brain NE was lower in Cu deficient rats (11). Lower NE was also observed in Cu-deficient lambs (12). Analyses in Cu-deficient rat and mouse brains detected lower NE concentrations in all regions except hypothalamus (13–15). Cu-deficient rodents exhibit elevated DA in brain regions enriched in noradrenergic neurons (14, 15). Collectively, these data provide strong support for limiting DBM activity in brain following Cu deficiency.

However, direct DBM enzyme assay of whole brain homogenates demonstrated higher activity in mottled mice (10, 16) and higher activity following dietary Cu deficiency in mice (16) and rats (15). Regional analyses of six rat brain areas confirmed and extended these observations that DBM activity, measured *in vitro*, was higher in brains of Cu-deficient rats (17). Thus, in brain a paradox exists. On the basis of metabolite levels, DBM activity is lower, but on the basis of direct assay, DBM activity is higher following Cu deficiency.

DBM and catecholamine levels have also been studied in adrenal gland following Cu deficiency with mixed outcomes. Hesketh reported lower NE levels in adrenals of Cu-deficient rats and cattle (18, 19). Elevated DBM activity was measured in rat adrenal homogenates following postnatal Cu deficiency (18–20). In young male rats, following perinatal Cu deficiency, adrenal DBM activity was also elevated (15). However, adrenal NE was not lowered by Cu deficiency in similar rats (20). Following postnatal Cu deficiency in rats, adrenal NE is sometimes unchanged and sometimes lower, whereas adrenal DA consistently is higher (8, 20). In Cu-deficient mottled blotchy mutant mice, total catecholamine content was unchanged but a 100% higher DBM activity was reported (21). Following dietary Cu deficiency, adrenal NE content was reported lower and DA higher in Cu-deficient mice (8). These facts suggest phenomena in adrenal gland similar to brain—a paradox between metabolite and enzyme assay data.

Assay of DBM requires addition of an agent, usually Cu^{2+} or N-ethylmaleimide (NEM) to inactivate an endogenous inhibitor. Regardless of the addition of NEM or Cu^{2+}, mouse brain DBM activity was higher in brindled mice or Cu-deficient mice than in controls (16). Mixing experiments also failed to detect any differences in the levels of activators or inhibitors between Cu-deficient and Cu-adequate rats (20). Protein levels of DBM have not been measured following Cu deficiency.

Recent experiments on brain and adrenal gland in two models of dietary Cu deficiency, a postnatal model studying the impact of postweaning Cu deficiency and a perinatal model studying the impact of gestational–lactational Cu deficiency, detected a robust increase in the steady-state DBM mRNA levels in Cu-deficient rats (20). This suggests that the elevated DBM activity, reported previously, might be due to increased formation of DBM protein. However, transcript levels do not always reflect protein levels following Cu deficiency. For example, Chen *et al.* showed that the mRNA for metallothionein (MT) in liver of Cu-deficient rats was elevated 75-fold compared to Cu-adequate rats, but MT protein was unchanged (22). Following Cu deficiency another group reported a 1.5-fold increase in olfactory bulb mRNA for neuropeptide Y but were unable to detect changes in immunoreactive peptide (23). The purpose of the present experiments was to test the hypothesis that increased DBM protein levels accompany dietary Cu deficiency. A second purpose was to assess the levels of TM following Cu deficiency since DBM and TM are often coordinately regulated.

Methods and Materials

Experimental Animals and Diets. Sperm-positive Sprague Dawley rats were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN). Rats received one of two dietary treatments, copper-deficient or copper-adequate, consisting of a Cu-deficient purified diet (Teklad Laboratories, Madison, WI) and either low-Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was formulated according to the AIN-76A diet 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; AIN-76A vitamin mix, 10; DL-methionine, 3; choline bitartrate, 2; and ethoxyquin, 0.01. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained 0.44 mg Cu/kg and 47 mg Fe/kg by chemical analysis. Offspring and dams on the Cu-deficient treatment drank deionized water, whereas Cu-adequate treatment groups drank water that contained 20 mg Cu/l by adding $CuSO_4$ to the drinking water. Rats were given free access to diet and drinking water. All animals were maintained at 24°C with 55% relative humidity on a 12-hr light cycle (0700–1900 hr). All protocols were approved formally by the University of Minnesota Institutional Animal Care and Use Committee.

Pregnant dams were placed on the Cu-deficient treatment 7 days after they were identified as sperm-positive. Two days following parturition litter size was adjusted to eight pups. Offspring were weaned when 21 days old, placed in stainless steel cages, and maintained on the same treatment as their respective dams for an additional 9 days. A total of 12 litters (6 Cu-adequate and 6 Cu-deficient) were sampled. This paradigm is similar to that used previously to study neurochemical changes in young rats (15). Males and females were killed on consecutive days.

Sample Collection. Offspring of dams were sampled at age 30 days. Blood samples were drawn into heparinized microhematocrit tubes from trunk blood following decapitation after light ether anesthesia. A small aliquot was also removed for hemoglobin analysis. Additional blood was collected in plastic tubes and allowed to clot. Plasma and serum were obtained by centrifugation. Livers were removed, rinsed with deionized water, and weighed and a portion was processed for metal analysis. Adrenal glands were removed, weighed to the nearest mg, and quick-frozen in liquid nitrogen. Brains were removed, and the cerebellum and hypothalamus were weighed and quick-frozen. Cerebella were analyzed for Cu and the hypothalamus for DBM, TM, and Cu,Zn-superoxide dismutase (SOD).

Chemical Analyses. Portions of liver, cerebella, and 1-g samples of diets were wet-digested with 4 ml of concentrated HNO_3 (AR select grade, Mallinckrodt, St. Louis, MO), and the residue was brought to 4.0 ml with 0.1 mol/l HNO_3. Samples were then analyzed for Cu and Fe by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT). Total protein content of adrenal and hypothalamus extracts was determined by analysis using a modified Lowry method with bovine albumin as a reference (24).

Enzyme Assays. DBM (EC 1.14.17.1) activity of adrenal gland and hypothalamus was determined spectrophotometrically by measuring conversion of tyramine to octopamine as described previously (16). The endogenous inhibitor of DBM activity was inactivated by 25 mmol/l N-ethylmaleimide (NEM) rather than Cu. Pairs of adrenal glands or brain tissues were homogenized for 30 sec in 9 vol of 0.05 mol/l potassium phosphate (pH 7.0) with a Tissumizer and microprobe (SDT-080EN, Tekmar Co., Cincinnati, OH). Homogenates were diluted in 0.005 mol/l potassium phosphate (pH 7.0) containing 0.2% Triton X-100 and centrifuged at 6,500*g* for 10 min. The phosphate–Triton buffer was stored in an acid-washed bottle containing 1 g of Chelex 100 (Bio-Rad Laboratories Inc., Hercules, CA) suspended within dialysis tubing. DBM activities were expressed per milligram of total protein. Activity of SOD (EC 1.15.1.1) was measured spectrophotometrically by monitoring inhibition of pyrogallol autoxidation at 320 nm as described previously (25).

Northern Blot Analysis. Total adrenal RNA was isolated from quick-frozen samples using a modified guanidine thiocyanate/phenol/chloroform procedure described in detail elsewhere (20). Total RNA was size fractionated on 1.5% agarose gels containing 2 mol/l formaldehyde and 0.02 mol/l sodium phosphate (pH 7.0) and transferred to Nytran Plus® membranes (Schleicher & Schuell, Keene, NH) by upward capillary transfer. Positions of 28S and 18S ribosomal RNA were marked after transfer. The membranes were then hybridized overnight with a purified ^{32}P random-primed 2.2-kb probe for rat DBM (1.7 ng/ml) (26). Membranes were washed several times and exposed to film (20). After autoradiography, membranes were stripped and rehybridized with a ^{32}P-labeled 1.2-kb probe for mouse 18S ribosomal RNA (Ambion, Austin, TX) to verify equal loading and transfer of RNA. Images of autoradiograms were captured using Gel Doc 1000 (Bio-Rad) interfaced with a Macintosh PPC computer, and band density profiles were integrated using the manufacture's software, Molecular Analyst (Bio-Rad).

Western Blot Analysis. Protein extracts from adrenal gland and hypothalamus were prepared in 0.05 mol/l potassium phosphate (pH 7.0) containing 0.2% Triton X-100 and mammalian protease inhibitor cocktail (Sigma P8340). Homogenates were centrifuged 1,000*g* for 10 min at 4°C to remove debris. Protein concentration was determined and samples diluted 1:4 with Laemmli sample buffer (0.0625 mol/l Tris (pH 6.8), 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.00125% bromophenol blue) and heated (boiling water bath 5 min) prior to loading. Proteins were size fractionated on 10% SDS-PAGE gels and electroblot transferred to 0.2 μM nitrocellulose membranes (Protran, Schleicher & Schuell).

Membranes were stained with Ponceau S (Sigma Chemical Co.) to ensure equal loading and to locate molecular weight markers. Immunoblotting was carried out at room temperature. Membranes were incubated with blocking solution (0.05 mol/l Tris (pH 8.0), 2 mmol/l $CaCl_2$, 0.08 mol/l NaCl, 5% non-fat dry milk, 0.2% Tween-20, and 0.02% sodium azide) with shaking for 45 min. Membranes were then incubated overnight or for 3 hr in seal-a-meal bags with primary antibody diluted 1:600 for DBM and 1:1,500 for TM, in TBST (0.05 mol/l Tris (pH 8.0), 0.9% NaCl, 0.1% Tween-20) and 1% blocking solution. Antibodies for DBM, rabbit anti-human DBM (AB 1538), and for TM, rabbit anti-rat TM (AB 152), were purchased commercially (Chemicon International Inc., Temecula, CA). For hypothalamus membranes, DBM was also probed using rabbit anti-rat DBM diluted 1:1,500 with antiserum against recombinant rat DBM (27). Following primary antibody incubation, membranes were washed with TBST (3 times for 5 min each), then were incubated for 1 hr with secondary antibody, donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham NA 934), diluted 1:5,000 in TBST. Membranes were then washed with TBST (3 times for 5 min each) and TBS (5 min). Detection measured chemiluminescence (ECL™, Amersham, Piscataway, NJ) exposing membranes to film (Kodak X-OMAT LS). Densitometry was performed using the manufacture's software (Bio-Rad). Size of the immunoreactive bands was estimated from regression analysis using standard peptides (Bio-Rad) log-[molecular weight] versus protein distance migrated.

Following primary target protein detection, membranes were directly reprobed for actin to ensure even sample loading and protein transfer (28). Membranes, previously probed for DBM and TM, were wrapped in plastic overnight at room temperature or up to 6 months at –20°C. They were washed with TBST for 10 min. and incubated with primary

antibody, mouse anti-chicken actin (MAB1501R, Chemicon International Inc.), as described above at a 1:10,000 dilution. After being washed, secondary antibody incubation and detection was performed as above with peroxidase conjugated goat anti-mouse IgG (Pierce 31434ZZ, Rockford, IL) at a 1:10,000 dilution.

Statistical Analyses. The effect of maternal Cu intake on offspring characteristics were analyzed by factorial ANOVA and significant interaction terms by Fisher's PLSD test, $\alpha = 0.05$ (Statview 4.5, Abacus Concepts, Inc., Berkeley, CA). Student's *t*-test was used when comparing data in which diet was the only variable, $\alpha = 0.05$.

Results

Following perinatal copper deficiency a number of characteristics indicated that the offspring subjected to the copper-deficient paradigm were indeed copper-deficient (Table I). One male and one female from each litter were sampled. Compared to copper-adequate females (+CuF) and males (+CuM) copper-deficient females (–CuF) and males (–CuM) were smaller. Females, as expected, were smaller than males at age 30 days. Relative weight of adrenal glands was not impacted by diet but by gender with female weights higher than male weights. The relative weights of the cerebellum and hypothalamus were impacted by both diet and gender, $P < 0.01$. Relative weights were higher in Cu-deficient compared to Cu-adequate rats and relative weights of females were greater than males (data not shown).

Hematocrit was lower in males compared to females and lower in Cu-deficient compared to Cu-adequate rats, though the degree of anemia suggested by lower hematocrit was modest (Table I). Liver Cu concentration was strongly impacted by diet and tended to be impacted by gender ($P = 0.053$). An interaction indicated that +CuF values were higher than +CuM values. However, it was clear that Cu-deficient offspring had major reductions in liver Cu (94% for –CuF and 89% for –CuM) (Table I). Liver Fe was not impacted by diet history or gender. Cerebellar Cu levels were 84% lower in –CuF and –CuM offspring compared to their +Cu controls. Cerebellar Fe, in nonperfused brains, was modestly lower in –CuF and –CuM rats compared to controls.

Adrenal glands from half the female offspring were assayed for DBM enzyme activity (Fig. 1). Compared to +CuF the DBM activity in the –CuF samples was 42% higher ($P < 0.05$). Protein concentration in the adrenal supernates was not altered by Cu deficiency. DBM activity was also measured on thawed homogenates of female offspring (Fig. 1). Activity is much lower in hypothalamus tissue compared to adrenal gland but activity of –CuF samples was higher than +CuF samples ($P < 0.001$).

Total RNA was isolated from adrenal glands of +Cu and –Cu 30-day-old male offspring and subjected to Northern hybridization analysis to assess DBM mRNA levels (Fig. 2). The steady-state concentration of DBM mRNA, normalized to the ribosomal 18S RNA signal, was 108% higher in the –Cu offspring compared to +Cu offspring. There were no significant differences in the 18S RNA signals between dietary treatment groups. The mean density ratio for –Cu/+Cu was 0.89.

Western immunoblot analysis was conducted on homogenates of female adrenal glands of 30-day-old rats. We originally tried to use the same extracts that were used for DBM enzyme activity. The blots for DBM seemed to work well; however, there was degradation evident when blotting for TM. Therefore, fresh homogenates containing protease inhibitors were used (Fig. 3). It was determined in other analyses, by linear regression, that the density observed was proportional to the protein loaded between 5 and 40 μg for the DBM signal ($r = 0.990$) and for the TM signal ($r = 0.996$). The commercial DBM antibody detected one major 76-kDa peptide in all six lanes, and it is readily apparent that a significantly stronger signal was present in the lanes representing –CuF compared to +CuF rats. The average enhancement was 45% (–Cu/+Cu = 1.45). The procedure was repeated with antibody against recombinant rat DBM with similar results (data not shown). The TM antibody detected one major 61-kDa peptide in all six lanes. The average enhancement was 39% (–Cu/+Cu = 1.39). Loading and transfer equivalency was evaluated by Ponceau S staining

Table I. Features of 30-Day-Old Female and Male Sprague Dawley Rats Following Perinatal Copper Deficiency[a]

Characteristic	Experimental group				ANOVA (*P*)		
	+CuF	–CuF	+CuM	–CuM	Diet	Gender	D × G[b]
Body weight (g)	80.8 ± 3.3[b]	65.0 ± 1.6[c]	104 ± 0.7[a]	77.7 ± 2.8[b]	0.0001	0.0001	0.0294
Adrenal/body (mg/g)	0.35 ± 0.02	0.36 ± 0.03	0.25 ± 0.02	0.25 ± 0.02	NS	0.0002	NS
Hematocrit	0.42 ± 0.01	0.38 ± 0.01	0.38 ± 0.01	0.36 ± 0.01	0.0003	0.0027	NS
Liver Cu (nmol/g)	164 ± 24[a]	9.51 ± 0.57[c]	111 ± 3.0[b]	12.2 ± 2.9[c]	0.0001	0.0527	0.0341
Liver Fe (μmol/g)	0.81 ± 0.14	0.96 ± 0.14	0.68 ± 0.11	0.73 ± 0.06	NS	NS	NS
Cerebellar Cu (nmol/g)	34.8 ± 3.2	5.49 ± 0.38	32.2 ± 1.1	5.12 ± 0.24	0.0001	NS	NS
Cerebellar Fe (μmol/g)	0.28 ± 0.03	0.21 ± 0.01	0.26 ± 0.02	0.23 ± 0.01	0.0287	NS	NS

[a] Values are means ± SEM ($n = 6$).
[b] For those characteristics with a significant interaction term (D × G) means were compared by Fisher's PLSD test.
Means not sharing a common superscript letter were different, $P < 0.05$. Details of the dietary treatments are listed in the Methods.

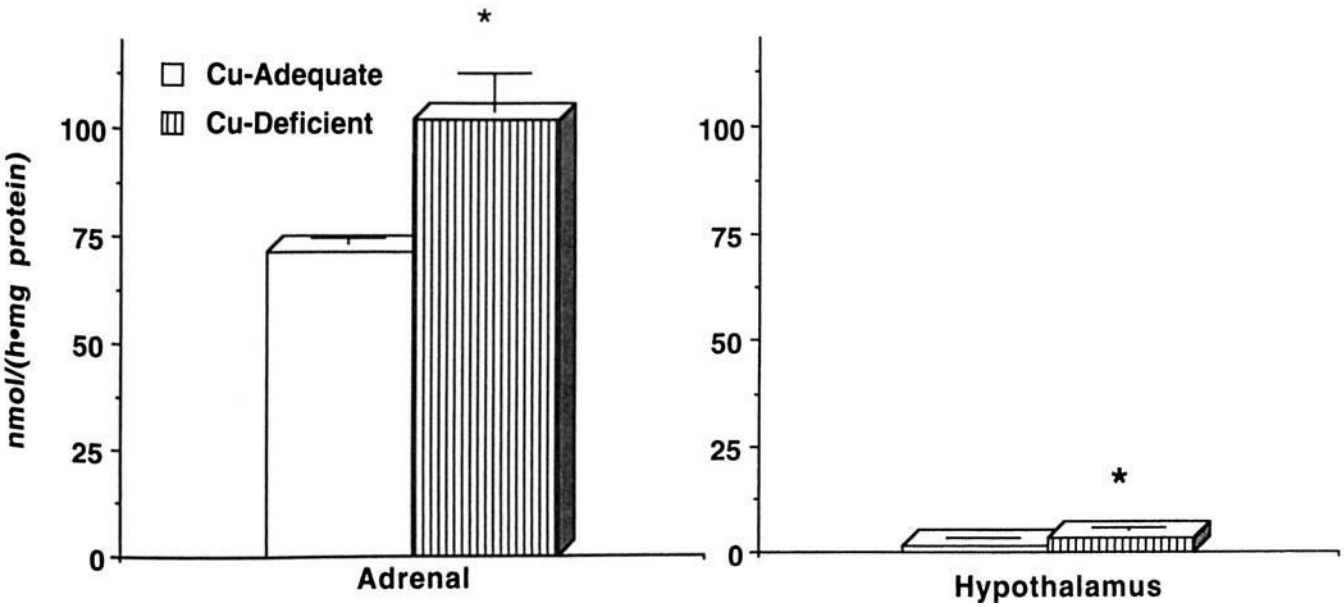

Figure 1. Dopamine β-monooxygenase activity (octopamine formation) of 30-day-old female Sprague Dawley rats after perinatal Cu deficiency. Bars represent means ± SEM (n = 3 adrenal, n = 4 hypothalamus). Means from –CuF were significantly different than means from +CuF, *$P < 0.05$.

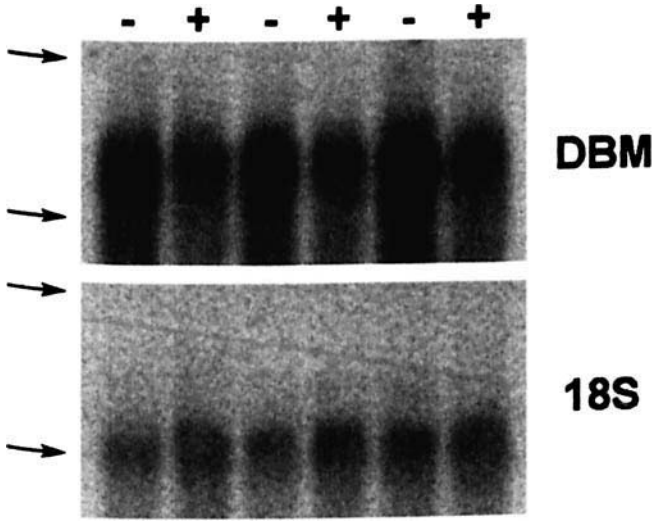

Figure 2. Northern blot hybridization analysis of rat adrenal total RNA (10 μg/lane) subjected to denaturing electrophoresis, capillary transfer, and binding to ^{32}P-labeled DNA probes specific for rat DBM and murine 18S ribosomal RNA. Arrows indicate migration position of 28S and 18S ribosomal RNA visualized with acridine orange. Lanes were loaded with RNA isolated from both adrenal glands of –CuM (–) and +CuM (+) 30-day-old rats following perinatal Cu deficiency. The DBM/18S density ratio was determined and the mean ± SEM for –CuM rats 2.38 ± 0.08 was higher than the ratio for +CuM rats 1.14 ± 0.09 ($P < 0.01$).

(data not shown). Additionally, each membranes was reprobed for actin. It is clear that the actin antibody detected a strong signal, with estimated size of 44 kDa, of equal intensity for each lane regardless of diet history. For the DBM membrane the density ratio for actin –Cu/+Cu was 0.85 and for the TM membrane the ratio was 0.99 ($P > 0.05$).

Western immunoblot analysis was also conducted on homogenates of hypothalamus from female 30-day-old rats (Fig. 4). The commercial antibody used for adrenal glands did not yield reproducible results. However, the anti-rat DBM antibody detected a 76-kDa peptide in all eight lanes. Density was not altered by diet history (–Cu/+Cu = 1.08). The TM antibody detected one major 61-kDa peptide in all lanes. We failed to detect any difference between treatment groups (–Cu/+Cu = 1.00). Actin reprobing confirmed equal intensities regardless of diet history (Fig. 4). The mean –Cu/+Cu actin density ratio for the DBM membrane was 1.00 and for the TM membrane 0.98 ($P > 0.05$). We were not successful in detecting DBM mRNA in hypothalamic RNA isolated and size-fractionated from four –CuM and four +CuM 30-day-old rats even after 8 days of exposure to film. The 18S signals on the reprobed membrane were equal and strong for all eight lanes (data not shown).

To assess the Cu status of the tissues used for Western blots, activity of the cuproenzyme SOD was measured on the same extracts (Fig. 5). Compared to activity of +Cu rats there was significantly lower ($P < 0.01$) SOD activity in –Cu rats. For adrenal gland the reduction averaged 50% and for hypothalamus 30%.

We were surprised that we could not detect a dietary impact on DBM protein levels in the hypothalamus, since enzyme activity was higher in –CuF extracts, so we carried out additional Western blots. Hypothalamic extracts from a single +CuF sample were loaded with odd number lanes receiving 36 μg and even lanes 30 μg. The 76-kDa DBM signal density detected was not significantly different; the odd/even ratio was 1.07 not 1.20 as expected. We repeated the experiment with extracts from a +CuF medulla/pons sample at a protein ratio of 1.67 (30 μg and 18 μg). The DBM density was different, $P < 0.05$, 1.39 ratio, but was lower than the predicted value (data not shown).

Our earlier diet work on perinatal rats studied mRNA

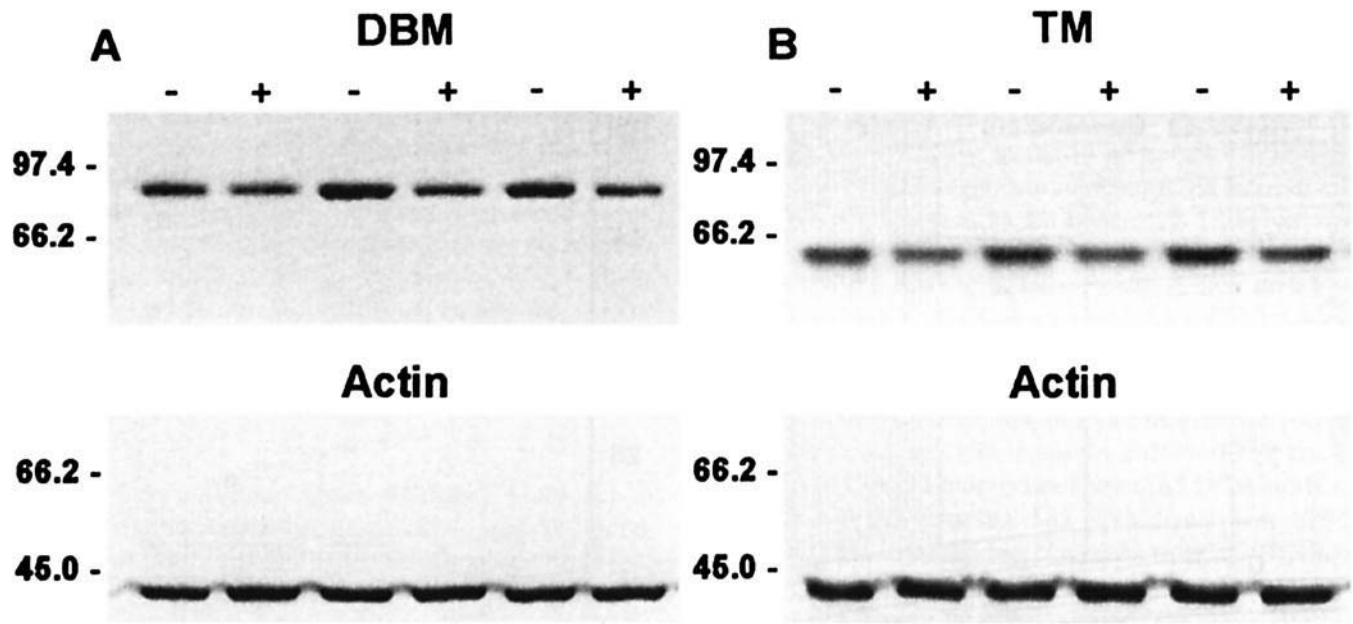

Figure 3. Western immunoblot of adrenal extracts detected with (A) rabbit anti-human dopamine β-monooxygenase (DBM) (Chemicon AB 1538) or (B) rabbit anti-rat tyrosine monooxygenase (TM) (Chemicon AB 152). Separate membranes were prepared. Each lane was loaded with 20 μg of protein. Three 30-day-old –CuF (–) and three +CuF (+) were analyzed. Secondary antibodies and chemiluminescent detection used protocols and reagents from Amersham (ECL™). Migration of phosphorylase b (97.4) and bovine serum albumin (66.2) are indicated. Mean ± SEM densities of lanes loaded with protein from –CuF adrenal glands were higher than for +CuF adrenal glands ($P < 0.05$) for both DBM (–CuF, 1.26 ± 0.11; +CuF, 0.87 ± 0.04) and TM (–CuF, 2.37 ± 0.15; +CuF, 1.70 ± 0.00). Blots were reprobed with mouse anti-chicken actin (Chemicon MAB1501R). Migrations of bovine serum albumin (66.2) and ovalbumin (45.0) are indicated. Mean densities for actin were not different between treatment groups ($P > 0.05$).

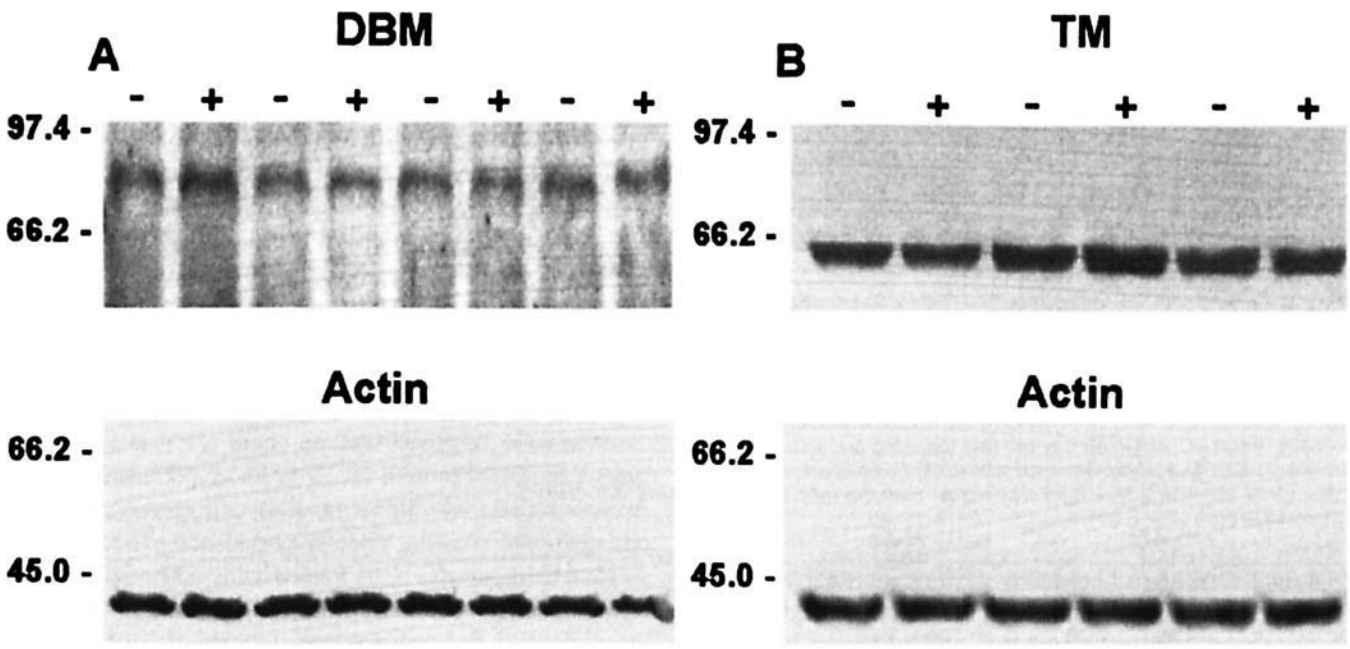

Figure 4. Western immunoblot of hypothalamus extracts detected with (A) rabbit anti-rat dopamine β-monooxygenase (DBM) (27) or (B) rabbit anti-rat tyrosine monooxygenase (TM) (Chemicon AB 152). Separate membranes were prepared. Each lane in panel A was loaded with 30 μg of protein, and in panel B with 60 μg of protein. Extracts from 30-day-old –CuF (–) and +CuF (+) were analyzed. Secondary antibodies and chemiluminescent detection used protocols and reagents from Amersham (ECL™). Migrations of phosphorylase b (97.4) and bovine serum albumin (66.2) are indicated. Mean ± SEM densities of lanes loaded with protein from –CuF hypothalamus extracts were not different than for +CuF extracts ($P > 0.05$) for DBM (–CuF, 40.1 ± 2.24; +CuF, 37.4 ± 3.21) or for TM (–CuF, 1.71 ± 0.09; +CuF, 1.72 ± 0.31). Blots were reprobed with mouse anti-chicken actin (Chemicon MAB1501R). Migrations of bovine serum albumin (66.2) and ovalbumin (45.0) are indicated. Mean densities for actin were not different between treatment groups ($P > 0.05$).

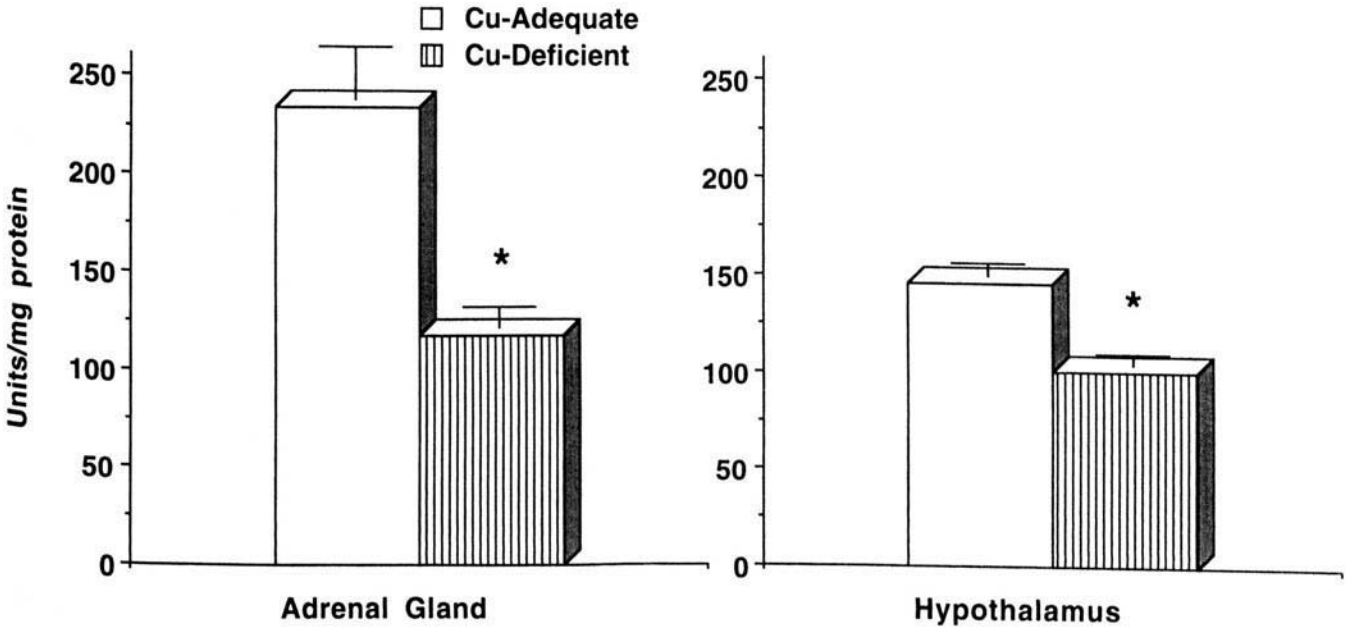

Figure 5. Cu,Zn-superoxide dismutase activity (inhibition of pyrogallol autoxidation) by extracts from 30-day-old female Sprague Dawley rats after perinatal Cu deficiency. Bars represent means ± SEM ($n = 3$). Means from –CuF were significantly different than means from +CuF, $^*P < 0.01$.

changes in the medulla/pons, thus, we conducted new studies using Western immunoblots from 24-day-old +CuF and –CuF rats ($n = 6$ each). Compared to +CuF rats the cerebral cortex Cu values were decreased 83%, 0.361 ± 0.025 μg/g compared to 2.12 ± 0.036 μg/g, $P < 0.01$. DBM activity was 41% higher in –CuF than +CuF samples, 4.32 ± 0.23 nmol/hr/mg protein compared to 3.06 ± 0.23. However, we failed to detect a difference in DBM density of the 76-kDa band (30 μg was loaded), –Cu/+Cu = 1.05. We also ran a separate gel and membrane for TM. There was no consistent or statistical difference between +CuF and –CuF medulla/pons samples in the twelve lanes for the 61-kDa band (10 μg protein was loaded). The signals were variable with an average –Cu/+Cu = 1.20.

Discussion

Following gestational and lactational restriction of dietary Cu, 30-day-old offspring exhibited features characteristic of severe Cu deficiency, including reduction in body weight and anemia. Also markedly lower liver and brain Cu concentrations were detected. These features are consistent with severe Cu deficiency and with features of 22-day-old offspring studied recently regarding DBM mRNA (20). Lower (50%) SOD activity confirms directly the Cu-deficient status of the adrenal glands in the current study. DBM adrenal enzyme activity when assayed *in vitro* under optimal conditions was higher in –CuF than +CuF rats in the current experiment. This work confirms previous studies in male rats using a similar paradigm (15). Northern analysis detected a broad mRNA band for DBM likely reflecting the failure to fractionate completely the 2.7- and 2.5-kb DBM transcripts produced by rats (26). Higher, 108%, steady-state concentrations of DBM mRNA were detected in –CuM compared to +CuM rats, suggesting higher DBM protein levels in adrenal glands of Cu-deficient rats. Western blot analysis of adrenal extracts of 30-day-old female rats revealed a single translation product of apparent size 76 kDa, consistent with the size reported by others for a glycosylated membrane-bound DBM (29, 30).

The 40% enhancement in DBM protein in –Cu rats implied by density of the lanes was similar to the enhancement in enzyme activity, suggesting, but not proving, that the higher *in vitro* enzyme activity of DBM in Cu deficiency is due to the presence of the higher DBM protein concentration. Previous mixing experiments failed to detect any abnormal amounts of activators in homogenates from Cu-deficient rats (20). Further support for higher DBM protein is evident by the enhanced density (–Cu/+Cu = 2.0) of adrenal DBM peptides in Western blots of 49-day-old male Holtzman rats subjected to 30 days of postnatal Cu deficiency (data not shown). These Cu-deficient rats also have higher (–Cu/+Cu = 2.4) DBM enzyme activity and higher (–Cu/+Cu = 2.8) DBM mRNA concentrations (20). Collectively these data strongly suggest that adrenal tissue from Cu-deficient rats contains higher concentrations of DBM protein than Cu-adequate rats. The enhancement in DBM mRNA in both models of Cu deficiency seem to exceed the enhancement in DBM protein levels; however, they were determined on different animals and in opposite genders in the case of the current studies. Adrenal extracts from 30-day-old –CuF rats contained higher TM levels detected by Western immunoblot analysis compared to +CuF extracts. The coding sequence of rat TM mRNA predicts a peptide of 56 kDa (31). However, we detected a single band of appar-

ent size equal to 61 kDa. This is consistent with prior work by others using a different antibody to rat TM (32).

The chemical signal that results in enhanced adrenal DBM and TM has not been identified. These proteins often change in concert because they share common promoter elements and respond to cAMP, glucocorticoids, and other chemokines (33–35). A logical hypothesis would be that Cu deficiency leads to elevated TM and DBM synthesis due to decreased levels of norepinephrine (NE). Epinephrine levels in adrenal gland of Cu-deficient rodents are not altered (8, 20). Reserprine, a drug that depletes adrenal catecholamines, leads to induction of adrenal DBM and TH (26, 36).

However, the data in Cu deficiency do not always support this hypothesis. Rats, similar to those studied in the current experiments, were shown to have no decrease in adrenal NE following Cu deficiency even though DBM mRNA was elevated markedly (20). Sabban *et al.* communicated similar findings (37). Moreover, mottled brindled mice, characterized by Cu deficiency because of a mutation in the ATP7A Cu efflux transporter gene, were found to have no alterations in adrenal DBM or TM activity despite a 42% reduction in adrenal catecholamines (21). The only consistent alteration in a catecholamine following Cu deficiency associated with elevated DBM is the robust increase in dopamine (DA). Thus, further research will be necessary to elucidate the signal that enhances DBM synthesis in Cu deficiency.

Reserpine treatment of rats is also associated with elevated TM and DBM in brain stem (38). Brain stems of –Cu rats contain lower NE and higher DA than +Cu offspring (15). Previously, higher DBM enzyme activity was reported in several rat brain regions, including hypothalamus and medulla/pons, for both –CuF and –CuM offspring (17). The –CuF rats in the current studies also had higher brain DBM activities than Cu-adequate controls. Thus, it was unexpected that we were unable to detect differences in DBM protein levels by Western immunoblot in hypothalamus or medulla/pons extracts from –CuF compared to +CuF rats. The DBM immunoblot procedure does not have the same sensitivity as the DBM assay, and the changes in brain DBM are less than in adrenal gland. In –CuF rats the medulla/pons DBM mRNA elevation was approximately 50% (20). However, it may also be possible that the apparent higher DBM activity is due to something besides higher DBM protein levels.

It should be pointed out, however, that despite the presence of higher brain DBM enzyme activity, mRNA, and, perhaps, protein, the functional activity of the enzyme appears to be lower rather than higher. The concentration of NE is lower and that of DA, the precursor, is higher in those Cu-deficient rodent brain regions innervated by noradrenergic neurons (14, 15). The lower NE, we hypothesize, is because the intracellular Cu is not sufficient to bind to apo-DBM to act as a cofactor. DBM undergoes facile Cu exchange in contrast to other cuproenzymes (39). The higher DA could be a combination of slower conversion by limiting DBM and enhanced synthesis by higher TM activity. These alternative hypotheses require further testing.

The current Cu-deficient studies in rats found no change in hypothalamus or medulla/pons TM protein levels by immunoblot analysis, whereas previous work in rats detected lower forebrain TM activity (40). In Cu-deficient brindled mice, higher TM activity in brain was detected in experiments following labeled tyrosine (9). Additional research on copper and catecholamine metabolism are needed to clarify these somewhat divergent observations on TM. It does seem that both brain stem TM and DBM change in response to stress (27). Restriction of Cu can be thought of as a physiological stress.

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1. Prohaska JR. Biochemical functions of copper in animals. In: Prasad AS, Ed. Essential and toxic trace elements in human health and disease. New York: Alan R. Liss, Inc., pp105–124, 1988.
2. Prohaska JR. Biochemical changes in copper deficiency. J Nutr Biochem **1**:452–461, 1990.
3. Friedman S, Kaufman S. 3,4-Dihydroxyphenylethylamine β-hydroxylase. Physical properties, copper content, and role of copper in the catalytic activity. J Biol Chem **240**:4763–4773, 1965.
4. Thomas SA, Matsumoto AM, Palmiter RD. Noradrenaline is essential for mouse fetal development. Nature **374**:643–646, 1995.
5. Gary T, Robertson D. Lessons learned from dopamine β-hydroxylase deficiency in humans. News Physiol Sci **9**:35–39, 1994.
6. Missala K, Lloyd K, Gregoriads G, Sourkes TL. Conversion of ^{14}C-dopamine to cardiac ^{14}C-noradrenaline in the copper-deficient rat. Eur J Pharmacol **1**:6–10, 1967.
7. Prohaska JR, Heller LJ. Mechanical properties of the copper-deficient rat heart. J Nutr **112**:2142–2150, 1982.
8. Prohaska JR, Bailey WR, Gross AM, Korte JJ. Effect of dietary copper deficiency on distribution of dopamine and norepinephrine in mice and rats. J Nutr Biochem **1**:149–154, 1990.
9. Hunt DM, Johnson DR. An inherited deficiency in noradrenaline biosynthesis in the brindled mouse. J Neurochem **19**:2811–2819, 1972.
10. Hunt DM. Primary defect in copper transport underlies mottled mutants in the mouse. Nature **249**:852–854, 1974.
11. Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: A possible model for Menkes' steely-hair disease. J Neurochem **23**:91–98, 1974.
12. O'Dell BL, Smith RM, King RA. Effect of copper status on brain neurotransmitter metabolism in the lamb. J Neurochem **26**:451–455, 1976.
13. Feller DJ, O'Dell BL. Dopamine and norepinephrine in discrete areas of the copper-deficient rat brain. J Neurochem **34**:1259–1263, 1980.
14. Prohaska JR, Bailey WR. Persistent regional changes in brain copper, cuproenzymes and catecholamines following perinatal copper deficiency in mice. J Nutr **123**:1226–1234, 1993.
15. Prohaska JR, Bailey WR. Regional specificity in alterations of rat brain copper and catecholamines following perinatal copper deficiency. J Neurochem **63**:1551–1557, 1994.
16. Prohaska JR, Smith TL. Effect of dietary or genetic copper deficiency on brain catecholamines, trace metals and enzymes in mice and rats. J Nutr **112**:1706–1717, 1982.
17. Prohaska JR, Bailey WR. Alterations of rat brain peptidylglycine α-amidating monooxygenase and other cuproenzyme activities following perinatal copper deficiency. Proc Soc Exp Biol Med **210**:107–116, 1995.

18. Hesketh JE. Catecholamine synthesis in the adrenal medulla of cattle deficient in copper. Biochem Soc Trans **8**:342, 1980.
19. Hesketh JE. The effect of nutritional copper deprivation on the catecholamine content and dopamine-β-hydroxylase activity of rat and cattle adrenal glands. Gen Pharmacol **12**:445–449, 1981.
20. Prohaska JR, Brokate B. Copper deficiency alters rat dopamine β-monooxygenase mRNA and activity. J Nutr **129**:2147–2153, 1999.
21. Hunt DM. Catecholamine biosynthesis and the activity of a number of copper-dependent enzymes in the copper deficient mottled mouse mutants. Comp Biochem Physiol C **57**:79–83, 1977.
22. Chen Y, Saari JT, Kang YJ. Copper deficiency increases metallothionein-I mRNA content selectively in rat liver. J Nutr Biochem **6**:572–576, 1995.
23. Rutkoski NJ, Fitch CA, Yeiser EC, Dodge J, Trombley PQ, Levenson CW. Regulation of neuropeptide Y mRNA and peptide concentrations by copper in rat olfactory bulb. Brain Res Mol Brain Res **65**:80–86, 1999.
24. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87**:206–210, 1978.
25. Prohaska JR. Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. J Nutr **113**:2048–2058, 1983.
26. McMahon A, Geertman R, Sabban EL. Rat dopamine β-hydroxylase: Molecular cloning and characterization of the cDNA and regulation of the mRNA by reserpine. J Neurosci Res **25**:395–404, 1990.
27. Serova LI, Nankova BB, Feng Z, Hong JS, Hutt M, Sabban EL. Heightened transcription for enzymes involved in norepinephrine biosynthesis in the rat locus coeruleus by immobilization stress. Biol Psychiatry **45**:853–862, 1999.
28. Liao J, Xu X, Wargovich MJ. Direct reprobing with anti-β-actin antibody as an internal control for western blotting analysis. BioTechniques **28**:216–218, 2000.
29. Sabban EL, Greene LA, Goldstein M. Mechanism of biosynthesis of soluble and membrane-bound forms of dopamine β-hydroxylase in PC12 pheochromocytoma cells. J Biol Chem **258**:7812–7818, 1983.
30. Sabban EL, Goldstein M, Greene LA. Regulation of the multiple forms of dopamine β-hydroxylase by nerve growth factor, dexamethasone, and dibutyryl cyclic AMP in the PC12 pheochromocytoma cell line. J Biol Chem **258**:7819–7823, 1983.
31. Grima B, Lamouroux A, Blanot F, Biguet NF, Mallet J. Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc Natl Acad Sci U S A **82**:617–621, 1985.
32. Haycock JW. Multiple forms of tyrosine hydroxylase in human neuroblastoma cells: Quantitation with isoform-specific antibodies. J Neurochem **60**:493–502, 1993.
33. McMahon A, Sabban EL. Regulation of expression of dopamine β-hydroxylase in PC12 cells by glucocorticoids and cyclic AMP analogues. J Neurochem **59**:2040–2047, 1992.
34. Lamouroux A, Houhou L, Biguet NF, Serck-Hanssen G, Guibert B, Icard-Liepkalns C, Mallet J. Analysis of the human dopamine β-hydroxylase promoter: Transcriptional induction by cyclic AMP. J Neurochem **60**:364–367, 1993.
35. Kumer SC, Vrana KE. Intricate regulation of tyrosine hydroxylase activity and gene expression. J Neurochem **67**:443–462, 1996.
36. Bhagat BD, Misra CH. Catecholamine depletion as a factor in the induction of adrenal tyrosine hydroxylase. Gen Pharmacol **8**:79–82, 1977.
37. Sabban EL, Failla ML, McMahon A, Seidel KE. Dietary copper can regulate the level of mRNA for dopamine β-hydroxylase in rat adrenal gland. FASEB J **5**:A1453, 1991.
38. Reis DJ, Joh TH, Ross RA. Effects of reserpine on activities and amounts of tyrosine hydroxylase and dopamine-β-hydroxylase in catecholamine neuronal systems in rat brain. J Pharmacol Exp Ther **193**:775–784, 1975.
39. Skotland T, Flatmark T. Dopamine β-monooxygenase. Binding to apoenzyme and rapid exchange in holoenzyme of ^{64}Cu studied with high-performance size-exclusion gel chromatography. Eur J Biochem **132**:171–175, 1983.
40. Morgan RF, O'Dell BL. Effect of copper deficiency on the concentrations of catecholamines and related enzyme activities in the rat brain. J Neurochem **28**:207–213, 1977.