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Superoxide Dismutase Activity in the Crinkled Mutant Mouse: Ameliorative Effects of Dietary Copper Supplementation (40636)

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Superoxide dismutase (S.O.D.) ($O_2^{\cdot -}$ – $O_2^{\cdot -}$ oxidoreductase EC 1.15.1.1.) catalyzes the disproportionation or dismutation of free $O_2^{\cdot -}$ radicals to yield hydrogen peroxide and oxygen (1, 2): ($O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$). The discovery of the activity of the enzyme was made by McCord and Fridovich (1) who showed that a copper- and zinc-containing protein, erythrocuprein, isolated from bovine red blood cells could catalyze the dismutation of superoxide ions. The protein was then assigned the more correct name S.O.D. This type of S.O.D. is generally found in the cytoplasm of all higher eukaryotic organisms (3) regardless of the cell type analyzed (4, 5). In addition to the cupro–zinc enzyme found in the cytoplasm, a manganese-containing enzyme having superoxide dismutase activity is found in the mitochondria of eukaryotic cells (3). It has been proposed that the physiological function of these enzymes is to protect oxygen-metabolizing organisms against the potentially deleterious effects of superoxide free radicals (6). The activity of cupro–zinc S.O.D. has been reported to be influenced by the level of dietary copper as inadequate levels of this trace element in the diet were associated with reduction of enzyme activity in swine (7) and in rats (8, 9).

The mouse homozygous for the autosomal recessive gene crinkled (*cr*) has several phenotypic characteristics similar to those seen in animals fed copper-deficient diets. The gene is characterized by decreased survival during the early postnatal period, alterations in skin thickness and pigmentation, decreased and retarded development of the hair bulbs, and neurological abnormalities (10, 11). Crinkled mice also show low liver copper concentrations from Day 18 of gestation to 21 days of age, but not thereafter (12). Diets high in copper (500 ppm) during pregnancy and lactation ameliorate many of the phenotypic traits of the gene (11–13).

In the present study, the activity of the copper metalloenzyme, S.O.D., was measured in livers of crinkled mice fed both normal and high copper diets and compared with the activity of the enzyme in noncrinkled mice. Animals were examined at both 14 and 60 days of age, since liver copper concentration was low before 21 days of age but normal thereafter (12). We also measured liver cupro–zinc S.O.D. activity in protein- and zinc-deficient rat pups at 14 days of age as a control for morbidity, since both of these groups also show high postnatal mortality.

Materials and methods. Known heterozygotic male and female carriers of the gene *cr* in a hybrid background were mated overnight. At the beginning of pregnancy, verified by the presence of vaginal plugs, females were housed in individual cages and randomly assigned to one of three groups. In one group, animals were given a commercial pelleted stock diet (Purina Laboratory Chow, Ralston Purina Co., St. Louis, Mo.) containing 12 ppm copper (by analysis). A second group received a purified control diet containing 6 ppm copper (by analysis), and the third group was fed the same purified diet with the exception that it was supplemented with copper to a level of 500 ppm. The composition of the complete purified diet has been reported previously (13). Dams were fed these diets throughout the experimental period. Food and distilled water were provided *ad libitum*. Temperature was maintained at 68–72°F; lighting was on a 12-hr on–off basis.

One crinkled (*cr/cr*) and one noncrinkled (+/?) littermate control were killed from each litter at 14 days of age. Some litters had no female crinkled pups at 14 days of age and for this reason, only males were tested. The time of killing was restricted to 0800–0900 hr in order to minimize possible diurnal variations. The pups were anesthetized with ether, the thoracic cavity was exposed, the superior vena cava severed, and, in order to remove

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blood, saline solution was perfused into the right atrium until the extremities appeared pale. Livers were excised and weighed and a small portion was kept for determination of hepatic copper concentration while the remainder was homogenized in 0.32 *M* sucrose. To remove mitochondrial mangano-S.O.D., homogenates were centrifuged at 13,000 *g* for 30 min. The assay was then conducted on the supernatant.

Cupro–zinc superoxide dismutase activity was determined by its ability to inhibit the autooxidation of epinephrine to adrenochrome at pH 10.2, according to the method of Misra and Fridovich (14). The reaction was followed spectrophotometrically using a Gilford kinetic spectrophotometer at 480 nm. One unit of S.O.D. was taken as the concentration giving a 50% inhibition of the autooxidation of epinephrine. The unit activity was computed from the linear portion of the graph, plotting the reciprocal of the slope of the autooxidation of epinephrine against the reciprocal of homogenate added. Values are expressed as activity/g tissue wet wt.

For copper determination, samples of liver were wet ashed with 16 *N* nitric acid, concentrated by evaporation, and diluted with deionized distilled water. Diet samples were first dry ashed at 450°C for 24 hr and then treated as described above. Copper concentration was determined by atomic absorption spectrophotometry (Perkin–Elmer Model 370).

In another experiment, the effect of age was examined in mice fed the stock diet. At 60 days of age, one crinkled and one noncrinkled mouse from each litter were killed and treated as described above.

To test the effect of zinc or protein deficiency on cupro–zinc S.O.D. activity, rats of the Sprague–Dawley strain were used. Two groups of pregnant females received diets adequate in zinc (100 ppm) during gestation. At parturition, they were fed a purified diet containing adequate zinc (100 ppm), or the same purified diet deficient in zinc (less than 0.4 ppm). The complete composition of the diet was reported previously (15). At 14 days of age, rat pups from each group were killed and liver S.O.D. activity was determined as described above. In addition, plasma zinc was measured by atomic absorption spectrophotometry after dilution with distilled water.

Additional rats were fed during gestation purified diets which were either adequate in protein (24%), or were protein deficient (4%). After parturition, both groups were fed the diet adequate in protein (24%). Fourteen days postnatally, pups were taken from both groups and liver cupro–zinc S.O.D. activity was determined as previously described. The complete composition of the diet has been published (16).

Statistical analyses. The significance of the difference between arithmetic means was evaluated by Student's *t* test.

Results. In the litters of dams fed either the stock diet or the purified control diet, liver cupro–zinc S.O.D. activity was significantly lower in the crinkled mutants than in their littermate controls, by about 30%. However, in crinkled mutants from dams receiving the copper supplemented diet (500 ppm Cu), activity of the enzyme was normal, the same as their littermate controls (Table I). Furthermore, copper supplementation did not produce an increased activity of the enzyme in noncrinkled mice over the values found in mice from stock fed or purified control diet fed dams (Table I).

In the litters of dams fed either the stock diet or the purified control diet, liver copper concentration was significantly lower at 14 days of age in crinkled mutants than in their littermate controls (Table I). There was no significant difference in liver copper levels between crinkled and noncrinkled mice from dams fed the copper-supplemented diet (Table I). As with cupro–zinc S.O.D. activity, there was no significant difference in liver copper concentrations of noncrinkled mice in any of the dietary groups (Table I). At 60 days of age, crinkled mutants showed significantly lower liver cupro–zinc S.O.D. activity than their littermate controls while liver copper levels were similar (Table I).

Cupro–zinc superoxide dismutase activity was not found to be significantly different between zinc-sufficient and zinc-deficient rat pups, although the plasma zinc concentrations indicated a severe deficiency (Table II). Similarly, cupro–zinc S.O.D. activity was not depressed by prenatal protein deficiency (Table II).

Discussion. It is apparent that one of the

TABLE I. SUPEROXIDE DISMUTASE ACTIVITY AND COPPER CONCENTRATION IN LIVER OF CRINKLED (cr/cr) AND NONCRINKLED (+/?) MICE

Diet	Experimental group	N	Superoxide dismutase (units/g wet wt)	Copper (μg/g wet wt)
Stock (12 ppm Cu)	14 days old			
	+/?	4	691 ± 109[a]	22.8 ± 1.2[a]
	cr/cr	4	388 ± 59[b]	17.5 ± 1.8[b]
Purified control (6 ppm Cu)	14 days old			
	+/?	4	590 ± 69[a]	25.4 ± 2.4[a]
	cr/cr	4	406 ± 41[b]	13.4 ± 2.1[b]
	60 days old			
	+/?	5	580 ± 40[a]	4.2 ± 0.5[c, d]
	cr/cr	5	420 ± 32[b]	4.4 ± 0.4[c, d]
Copper supplemented (500 ppm Cu)	14 days old			
	+/?	4	581 ± 77[a]	23.4 ± 2.0[a]
	cr/cr	4	650 ± 61[a]	28.3 ± 4.0[a]

[a, b, c] Values are expressed as mean ± SE of the mean. Values with different superscripts are significantly different, $P < 0.05$ (Student's t test).
[d] Data from three animals.

TABLE II. LIVER SUPEROXIDE DISMUTASE ACTIVITY AND PLASMA ZINC CONCENTRATIONS IN 14-DAY-OLD RATS

Diet	N	Superoxide dismutase (units/g wet wt)	Plasma (μg zinc/dl)[a]
100 ppm Zn	6	464 ± 34	116 ± 7
0 ppm Zn	6	448 ± 24	60 ± 5*
24% Protein	4	487 ± 33	—
4% Protein	4	488 ± 29	—

[a] Values are expressed as mean ± SE of the mean.
* Significantly lower than control at $P < 0.01$ (Student's t test).

phenotypic expressions of the gene *cr* is a lower than normal activity of the enzyme cupro–zinc S.O.D. To determine if the low activity of the enzyme in the crinkled mutant is the result of a mutation in the S.O.D. gene itself, we have examined the electrostatic properties of the cupro–zinc S.O.D. isoenzymes by isoelectric focusing. There was no difference found in the isoenzyme pattern between the crinkled and noncrinkled mouse (unpublished results), which suggests that the low activity of the enzyme in the crinkled animals is due to the mutants poor copper status, and not to a specific S.O.D. gene mutation. While there have been reports dealing with abnormal isoenzyme variants of S.O.D. in humans, it is not clear that the activity of the enzyme is altered in these individuals (17–19). We believe our report is the first to demonstrate a depression of S.O.D. activity in a genetic mutant and its increase to normal levels by means of dietary manipulation.

The two identified errors of copper metabolism in the crinkled mouse, low liver copper concentration and abnormal copper intestinal transport, are not detectable past the early postnatal period (12, 20). Since copper concentration in the liver was normal after 21 days of age, it was surprising that the low activity of cupro–zinc S.O.D. in the nonsupplemented mutant persisted beyond this time. This suggests that cupro–zinc S.O.D. activity may be permanently affected by the early nutritional environment of the animal. Similar sustained pertubations have been reported to occur for the enzymes 3-hydroxy-3-methylglutaryl coenzyme A reductase (21), acetyl-CoA carboxylase (22), alkaline phosphatase, and disaccharidase (23), in rats exposed to varying nutritional environments.

The finding of low liver copper concentrations in the nonsupplemented mutants, while liver copper concentration and cupro–zinc S.O.D. activity in the copper supplemented mutants were similar to littermate controls, supports the idea that low activity of the enzyme in the mutant is correlated with an abnormality in copper metabolism. The finding of normal levels of cupro–zinc S.O.D. activity in both zinc-deficient and protein-deficient rat pups argues against the hypothesis that low levels of cupro–zinc S.O.D. activity are a common sign in states of nutritional inadequacy. The important finding

that decreased copper levels in the mutant can be correlated with decreased cupro–zinc S.O.D. activity emphasizes the need to perform studies on other copper-dependent enzymes in this mutant. Since it has been proposed that low levels of S.O.D. activity can be deleterious to oxygen-metabolizing organisms (6), the combined effect of this insult together with decreased activities of other copper-dependent enzymes could explain the poor viability of the mutant.

Since the report of McCord and Fridovich (1) suggesting that the enzymatic function of the blue copper protein "erythrocuprein" could be to catalyze the dismutation of superoxide free radical anions, interest in the enzyme has been steadily mounting. While the enzyme is generally believed to have a protective function, its precise biological role is ill defined (24). One method of attempting to define better the role(s) of S.O.D. is to study it in normal or pathological situations where its activity is altered or in a state of flux. For example, an increase in S.O.D. activity has been found to precede rapid cell growth in *Escherichia coli* (25), while a decrease in S.O.D. activity with age has been suggested as a causative factor for age-related damage to cells in mice (26). Bonta has reported that S.O.D. activity in human cord blood is inversely related to gestational age, while a rapid increase in activity occurs shortly after birth, presumably triggered by the exposure of the neonate to high oxygen tensions. Failure of this process to occur has been suggested as part of the etiology of infant respiratory distress syndrome (27). Fluctuations in S.O.D. activity have also been correlated recently with chronic infection anemias in the dog (28), the occurrence of rheumatoid arthritis (29), diabetes (30), and constitutional infantile panmyelopathy in man (31).

It is obvious that a more complete understanding of the activity and control of S.O.D. is necessary, not only with regard to its role in normal development, metabolism, and aging, but also because of its putative involvement in several pathological conditions. The finding that cupro–zinc S.O.D. activity is depressed in the crinkled mutant, and that it can be increased to normal levels by dietary copper supplementation may allow this mutant to serve as a tool in studies on control mechanisms for the enzyme, as well as on the effects of its deficiency.

Summary. Liver cupro–zinc superoxide dismutase activity was lower in mice homozygous for the gene *crinkled* (*cr*) than in noncrinkled littermate controls (+/?), both at 14 and at 60 days of age. Liver copper concentration was also low at 14 days, but was normal at 60 days. Supplementation during pregnancy and lactation with a high level of dietary copper brought both cupro–zinc S.O.D. activity and copper concentration in the liver of mutant offspring to levels similar to those of nonmutant littermate controls. Liver cupro–zinc superoxide dismutase activity was not found to be influenced by a deficiency of either zinc or protein in the rat.

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