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Manganese deficiency and transcriptional regulation of mitochondrial superoxide dismutase in hepatomas

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The presumed involvement of the transition metals manganese and copper in the regulation of the expression of the Mn- and CuZn-containing superoxide dismutase genes has been investigated in normal and neoplastic tissues of the rat. Two hepatomas of the Morris line have been employed, the slow growing, highly differentiated 9618A and the fast growing, poorly differentiated 3924A. The data obtained indicate a control at the pretranslational level of the Mn-containing enzyme, presumably exerted by the manganese ion. The CuZn-containing superoxide dismutase is also regulated pretranslationally in the normal tissues examined and in the hepatoma 3924A. However, there is no indication for the involvement of the copper ion, which in the liver is mostly located in the cytosol bound to CuZnSOD, in such regulation. The possible role of a reduced redox state, concomitant to the manganese deficiency in hepatoma tissues, in the down regulation of Mn-containing superoxide dismutase is discussed.

Mn^{2+}-superoxide dismutase; SOD gene expression; Hepatoma; Manganese deficiency; Redox stress; Transition metal

1. INTRODUCTION

Interest in regulatory mechanisms of superoxide dismutases (SODs) expression is increasing greatly. It stems from (i) the main role sustained by these enzymes in the defense against deleterious reactive oxygen species (ROSs), presumably involved in a variety of pathophysiological conditions (such as inflammation [1], cardiovascular diseases [2], neoplastic transformation and growth [3–6], aging [7], etc.) and from (ii) the observation that the biosynthesis of these metalloproteins is generally subjected to a sort of modulation, depending on endogenous stimuli or environmental insults. For this reason SODs are now considered to belong to the family of the so-called stress proteins [8] and common or similar mechanisms could account for the response (i.e. induction of a restricted number of proteins) to various stress conditions, including oxidative injury.

Most of the work on the regulation of SODs, especially the manganese-containing SOD (MnSOD), has been performed in prokaryotic cells. Some models have been proposed, all indicating that these proteins are subject to rigorous control systems [9–13]. It now seems to be accepted that the MnSOD gene, at least in prokaryotes, is part of a complex genetic locus (*soxRS* Regulon) which controls the synthesis of nine proteins, up-regulated in response to superoxide stress [14–16]. However, the intimate mechanisms involved in the regulation of this locus and thus of MnSOD expression are yet poorly defined. In any case, the modulation of SOD synthesis is always exerted by changes in the redox status of the cell [17]. Another interesting peculiarity concerning SODs is that they are generally decreased in tumor cells, the MnSOD being more affected by such feature [3,18]. We have already reported [19] that Cu,Zn-containing CuZnSOD (cytoplasmic) and MnSOD (mitochondrial) activities are diminished in rat hepatomas. Synthesis of MnSOD is controlled pretranslationally, independent of the differentiation of the hepatomas. CuZnSOD, on the other hand, is down-regulated (post-)translationally in the highly-differentiated tumor (9618A) and pretranslationally in the poorly-differentiated one (3924A). In the present study we have investigated the role of the transition metals, manganese and copper, in the control of SODs gene expression. Morris hepatomas 9618A and 3924A, with the corresponding rat livers as control, and different rat tissues, in which both CuZn and MnSOD are physiologically expressed at different levels, have been utilized as experimental models. A preliminary account of this work has been given [20].

2. EXPERIMENTAL

2.1 *Materials*

Guanidine hydrochloride (ultra pure) was purchased from Sigma Chem. [α-^{32}P]dATP and oligo(dT)-cellulose columns were supplied by NEN Research Comp.; terminal deoxynucleotidyl transferase by

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Gibco-BRL. All other chemicals were molecular biology reagents or of analytical grade.

2.2 *Tumor and tissue preparations*

Morris hepatomas 9618A and 3924A [21], propagated in Buffalo and ACI/T inbred rats, respectively, were utilized as in [19]. The following tissues of normal male rats (200–220 g) of the ACI/T strain were used for enzyme assay, mRNA and metal quantification: liver, brain, spleen, thymus, lung, kidney and heart. Pooling was applied where necessary. Tumor and liver mitochondria were prepared according to the method of Pedersen et al. [22]. Proteins were estimated by the biuret procedure [23].

2.3 *RNA isolation*

RNA was isolated essentially according to Chirgwin et al. [24]. Tissue samples were homogenized in 10 volumes of 4 M guanidine thiocyanate for one minute at full speed using a Polytron tissue homogenizer. The homogenates were layered over 1.2 ml of sterile 6.75 M CsCl, 0.1 M EDTA (pH 7.0) and centrifuged in a Beckman SW50.1 rotor for 12–20 h at 36,000 rpm and 22°C. The subsequent collection and processing of the isolated RNA and the preparation of polyadenylylated [poly(A)+]RNA by the use of an oligo(dT)-cellulose column are specified in [19].

2.4 *Quantitative analysis of mRNAs*

Total and poly(A)+RNAs for the analysis of CuZnSOD and MnSOD messenger RNAs, respectively, were size-fractionated by formaldehyde-agarose (1.5%) gel electrophoresis and blotted onto nitrocellulose membranes. The pre-hybridization and hybridization conditions and the 36- and 33-oligomers used as probes, for CuZnSOD and MnSOD, respectively, were the same as in [19].

Two additional 30-mer deoxyoligonucleotides were employed in hybridization experiments for the detection of MnSOD mRNA. The sequences of these probes, chosen in the coding region of the rat MnSOD cDNA [25], were from Gly-127 to Gln-136 (5'-CTGTAAGCGACCTTGCTCCTTATTGAAGCC-3') and from Val-189 to Lys-198 (5'-CTTCTTGCAAACTATGTATCTTTGGCTAAC-3'). Stringency experiments were carried out by varying the temperature of the washing steps of the hybridized filters [26]. MnSOD mRNA in poly(A)+RNA Northern blots was quantitated according to [19].

2.5 *Enzyme assay*

CuZnSOD and MnSOD activities were evaluated on 48-h-dialyzed homogenates of tumor and normal tissue specimens as described in [19] by the method of inhibition of hematoxylin autoxidation to hematein [27] with the exception that MnSOD was measured in the presence of 1.5 mM cyanide.

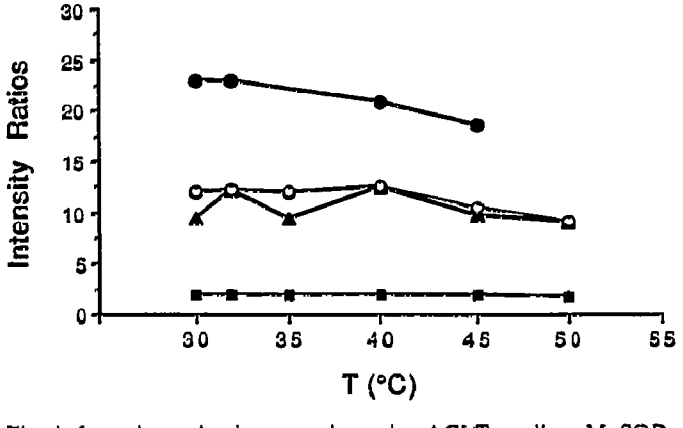

Fig. 1. Intensity ratios between the major ACI/T rat liver MnSOD message (0.85 kb) and the minor hybridization bands as a function of temperature. The ratios refer to bands in the Northern blots of poly(A)+RNA with sizes of 1.08 (■), 2.1 (▲), 3.0 (●) and 4.1 (○) kb. Their intensities were about 50 (1.08 kb), 10 (2.1 and 4.1 kb) and 5 (3.0 kb) percent with respect to the 0.85 kb band. The sequence of the 33-oligomer probe employed is reported in ref. 19.

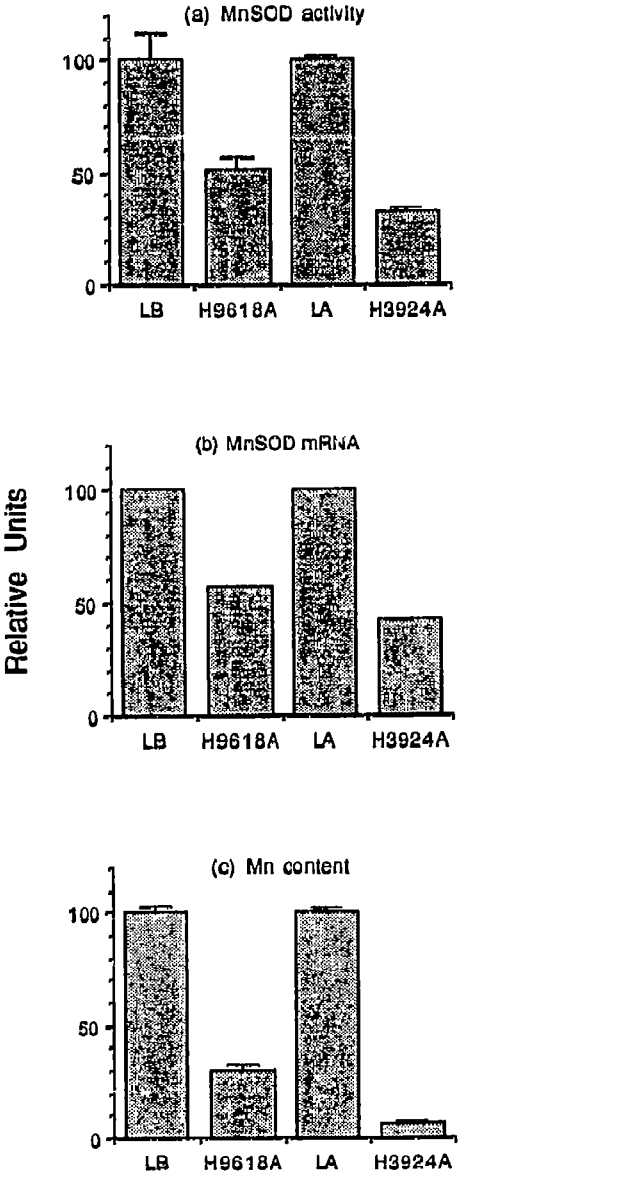

Fig. 2. Comparison between activity of MnSOD, concentration of its message and Mn content in the liver of inbred Buffalo (LB) and ACI/T (LA) rats and in hepatomas. Hepatoma values are normalized to those of normal livers. The absolute control values for the enzyme activity were: 843 ± 99 (3) (LB) and 673 ± 15 (4) (LA) μg/g dry weight. Those for the metal were: 9.4 ± 0.25 (9) (LB) and 9.2 ± 0.15 (12) (LA) μg/g dry weight. Enzyme activities in hepatomas are expressed as means ±S.E. of three experiments. Values of Mn content are means ±S.E. of 9 (hepatoma 9618A) and 12 (hepatoma 3924A) experiments. MnSOD mRNA levels correspond to the sum of the intensities of the two major hybridizing bands (≈ 1080 and ≈ 850 nt). The mRNA values were corrected for the variability of the polyadenylylated RNA content in each poly(A)+RNA preparation [19] and are means of 2 experiments for livers and one for hepatomas performed by using two concentrations of poly(A)+RNA in each experiment. The relative amount of message is 57% higher in LB with respect to LA [19].

Table I

Manganese content of whole tissue and mitochondria isolated from rat liver and hepatomas

Tissue source of mitochondria	Mn content		
	Whole tissue (ng/g dry weight)	Mitochondria	
		(ng/mg protein)	(ng/g whole tissue dry weight)
Liver Buffalo	9400 ± 250 (9)	27.2 ± 3.7 (6)	1809
Hepatoma 9618A	2850 ± 160 (9)	11.1 ± 0.4 (3)	201
Liver ACI/T	9190 ± 150 (12)	26.4 ± 2.2 (4)	1544
Hepatoma 3924A	610 ± 50 (12)	5.6 ± 1.2 (6)	112

Mitochondrial manganese in the whole tissue has been calculated on the basis of the amount of protein in the mitochondrial fraction after isolation. The contents of mitochondria were: 66.5 (LB), 18.1 (H9618A), 58.5 (LA) and 20 (H3924A) mg protein/g whole tissue dry weight (2-3 experiments). Value are means ±S.E. (number of observations). Determination of mitochondrial content of H3924A by enzymatic or spectral cytochrome oxidase assays on whole-cell suspensions gave similar results [29].

2.6 *Metal determination*

Metal concentrations were determined by atomic absorption using a Perkin-Elmer 272 spectrophotometer. Thin slices of each tissue sample were dried at 100°C, digested with 1 N nitric acid, and then analyzed for metal content.

3. RESULTS

In a previous study [19] we reported on the existence in rat liver of five distinct species of manganese superoxide dismutase mRNA. The two major messages have a size of about 850 and 1080 nt and the three minor ones are about 2100, 3000 and 4100 nt long. This observation is consistent with that of Ho et al. [28] who found six transcripts for rat lung MnSOD. All five bands hybridized with the three deoxyoligonucleotide probes, derived from the coding region of the rat enzyme cDNA (not shown). Moreover, as shown in Fig. 1, by varying the hybridization conditions as a function of temperature, the ratios between the intensity of the strongest band (0.85 kb) and that of each minor one remained fairly constant, except for the 3.0 kb species, owing perhaps to the weakness of its signal which was particularly difficult to detect at high temperatures. This observation is indicative of the specificity of all five transcripts whose nature is still to be investigated. They could be messages of two different genes plus forms of splicing intermediates of the MnSOD RNA [28].

As shown in Fig. 2, the activity of the Mn-containing enzyme is markedly diminished in the highly differentiated, slow-growing hepatoma 9618A and even more in the poorly differentiated, fast-growing 3924A, with a well corresponding decrease of the relative concentration of their transcripts. Interestingly, a parallel diminution of manganese content can be observed (see also Table I). Also for the other rat tissues examined (Fig. 3) a very good correlation between enzymatic activity, messenger RNA and manganese content must be highlighted. The only exception is the heart, in which, besides a high enzymatic and mRNA level, a low manganese content was detected. The thymus and lung, in addition to the heart, also show a much lower Mn concentration per MnSOD activity and mRNA than the other tissues. The decrease in manganese content observed in the whole tumor tissue reflects also the mitochondrial compartment (Table I). Indeed, the ion con-

Table II

CuZnSOD activity, mRNA and metal ion content of rat liver and hepatomas

Tissue	CuZnSOD (mg/g dry weight)	mRNA (arbitrary units)	Metal ions (μg/g dry weight)	
			Cu	Zn
Liver Buffalo	2.31 ± 0.13 (3)	100 ± 4.1 (3)	12.3 ± 0.33 (9)	114.6 ± 2.66 (9)
Hepatoma 9618A	1.21 ± 0.02 (3)	99.5 ± 7.3 (7)	11.3 ± 0.57 (9)	111.0 ± 4.77 (9)
Liver ACI/T	2.21 ± 0.10 (4)	100 ± 5.4 (3)	12.7 ± 0.39 (11)	115.3 ± 2.10 (9)
Hepatoma 3924A	0.36 ± 0.04 (3)	23 ± 2.7 (5)	11.4 ± 0.85 (12)	122.8 ± 3.83 (9)

mRNA values in hepatomas are expressed as percent of normal liver. The relative amount of mRNA is 21% higher in the liver of Buffalo than in that of ACI/T rats [19]. Values are means ±S.E. (number of observations).

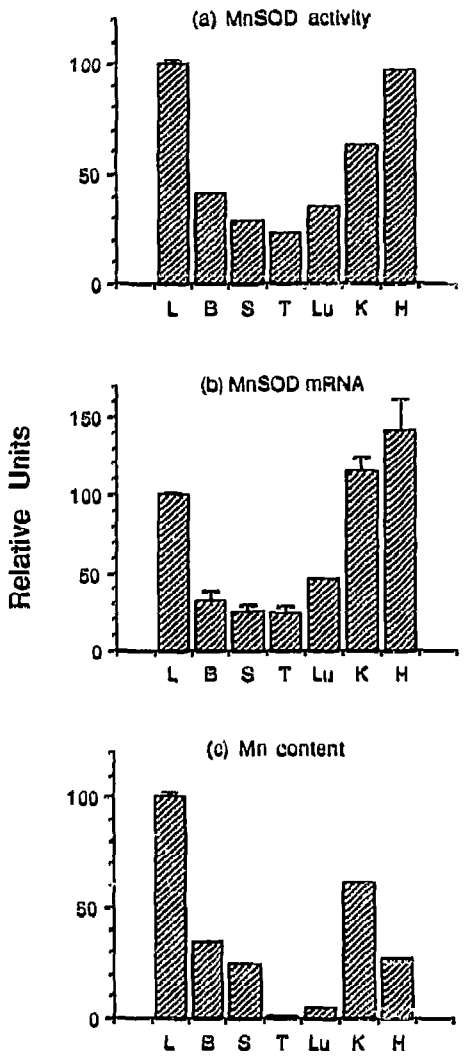

Fig. 3. Activities of MnSOD, concentrations of mRNA and contents of Mn in various tissues of ACI/T rats. Enzyme and Mn values are means of 2 to 4 experiments. Liver MnSOD is 664 ± 10.2 μg/g dry weight. The liver Mn content is equal to that reported in Fig. 2. MnSOD mRNA levels were calculated, as described in Fig. 2, on 3 to 4 samples. The values of the different tissues are normalized to that of liver. L, liver; B, brain; S, spleen; T, thymus; Lu, lung; K, kidney; H, heart.

tent of mitochondria isolated from the hepatomas 9618A and 3924A is about 40 and 20% of mitochondria from host livers, respectively. If then the mitochondrial manganese content is referred to the whole tissue, the difference between hepatic and hepatocarcinoma cells appears to be even more pronounced, because of the lower content of mitochondria of the latter cells (see also ref. [29]).

Table II reports the values of CuZnSOD activity, copper and zinc content and the levels of messenger RNA for the 9618A and the 3924A hepatomas and the respective Buffalo and ACI/T control livers. As reported previously, it is confirmed that the enzyme in the hepatoma 3924A is regulated at a pretranslational level, the enzyme activity and mRNA concentration being both lowered by about 80% with respect to the control tissue [19]. On the contrary, in the hepatoma 9618A the normal amount of CuZnSOD mRNA is preserved whereas the enzymatic activity is significantly diminished (about 50%). It is noteworthy that in both tumors the level of the metals, and in particular of copper, which is the transition metal responsible for the catalytic activity, are unchanged. The various rat tissues examined (Table III) are characterized by different CuZnSOD activities, strictly correlated with the levels of the RNA transcripts. Conversely, the copper contents show a poor correlation either with the enzymatic activities or the mRNA values. This observation clearly indicates that CuZnSOD, which unlike MnSOD is known to be constitutive [30], is physiologically under a transcriptional regulation of gene expression (cf. ref. [31]) and that the metal ion copper (70% of which is bound to the enzyme in the liver cytosol) is not involved in such regulation but only in the posttranslational induction of the dismutase activity (i.e. by activation, upon insertion of the metal, of the preexisting apoenzyme).

4. DISCUSSION

The most relevant observation of this study is that cells from both the hepatomas studied are characterized, in addition to a low MnSOD activity, which is transcriptionally regulated by low levels of mRNA, by a low content of manganese ions (Fig. 2 and Table I). Moreover, these three parameters gradually decrease in parallel with the degree of tissue differentiation. A loss of manganese to an extent similar to that of the hepatoma 3924A, has been reported by Ling et al. [32] in rat hepatoma As-30 and in six strains of mouse cancer cells. It is evident that the manganese deficiency itself does not posttranslationally down-regulate the enzyme activity, i.e. increased concentration of the apoenzyme, since the messenger RNA level is modulated to the same extent as the activity and the metal. Furthermore, owing to the fact that, besides MnSOD, manganese is present in tissues as the prosthetic element of many other proteins, it is unlikely that the MnSOD transcript alone regulates the intracellular content of the metal (i.e. all Mn is bound to MnSOD). Indeed we calculated, using data from Table I and Fig. 2, the amount of metal bound to MnSOD in liver (about 2 μg/g dry weight) which corresponds to 20% of the total manganese in the tissue and is close to that found in the mitochondrial fraction where the enzyme is localized. The metal ion, however, could directly or indirectly modulate the transcriptional level of the enzyme. Data from rat tissues (Fig. 3), showing that the levels of manganese agree well, with the exception of heart, thymus and lung (lower than expected), with those of mRNA and enzymatic activity, offer support to this hypothesis.

Table III

CuZnSOD activity, mRNA and metal ion content in various rat tissues

Tissue	CuZnSOD (mg/g dry weight)	mRNA (arbitrary units)	Metal ions (μg/g dry weight)	
			Cu	Zn
Liver	2.22 ± 0.11 (4)	100 ± 5.0 (5)	12.7 ± 0.39 (11)	115.3 ± 2.10 (9)
Brain	0.53 (2)	23.5 ± 2.3 (5)	11.6 (2)	66.7 (2)
Spleen	0.49 (2)	18.3 ± 1.4 (5)	6.2 (2)	105.8 (2)
Thymus	0.40 (2)	15.8 ± 1.9 (5)	2.9 (2)	100.7 (2)
Lung	0.43 (2)	16.0 ± 1.2 (5)	7.2 (2)	105.6 (2)
Kidney	1.85 (2)	99.1 ± 7.8 (6)	47.1 (2)	98.5 (2)
Heart	0.68 (2)	35.5 ± 4.0 (4)	23.3 (2)	79.1 (2)

Values (means ±S.E.) refer to tissues of inbred ACI/T rats.

An additional indication of a strict correlation between ion content, message concentration and enzyme activity of MnSOD derives from experiments on developing rat liver. In the first days after birth the three parameters range from 40 to 60% of the adult and reach the maximum in about two weeks (unpublished observations). MnSOD, contrary to CuZnSOD whose synthesis is constitutive, is inducible by physical agents and a large variety of exogenous and endogenous molecules. A partial list, in mammalian cells, includes X-ray irradiation [33], hyperoxia [34], redox-cycling substances such as paraquat [35], and treatment with 2,4-dinitrophenol [36], bacterial lipopolysaccharide and cytokines [37–43]. The molecular mechanism of MnSOD induction has not yet been elucidated, but it is probably mediated by an increase of oxygen radicals which might act on some 'sensor' molecule. Manganese can facilitate the generation, as a transition metal, of ROSs by speeding up the dismutation of O_2^- to H_2O_2 [44], and in this way it may indirectly promote the transcriptional induction of MnSOD. ROSs have been recently [45,46] implicated as responsible for the posttranslational activation of the NF-*k*B transcription factor which can rapidly induce the expression of genes involved in many cellular responses such as inflammatory, immune and acute phase reactions. The NF-*k*B activation, which involves the release of the inhibitory subunit I*k*B from the latent cytoplasmic form (p50-p65-I*k*B complex) [47], is prevented by antioxidants or metal chelators [45]. It has also been reported that binding of the AP-1 transcription factor to DNA may be induced by H_2O_2 [48]. The proposed functional role of ROSs would then support a unifying hypothesis, according to which such molecules could act as second messengers in the mechanism of action of MnSOD inducers, among which may be transition metals. Cellular signalling via ROSs could also account for the behavior of rat heart which exhibits, besides a rather low manganese content, high levels of MnSOD mRNA and activity, possibly related to an active mitochondrial, oxyradical generating, electron transport system.

With regard to the CuZn-containing superoxide dismutase, it appears that in neoplastic tissue copper content does not affect the enzyme expression, as its concentration is the same as that of the normal tissue. SOD-bound Cu in liver is about 70% of the total metal in the tissue (about 9 μg/g dry weight). The decrease of CuZnSOD activity in hepatomas does not influence their Cu content, suggesting an increase of the free form of the metal. The enzyme is regulated (post-)translationally, perhaps through a catabolic mechanism, in the highly differentiated hepatoma and this mechanism is overtaken by pretranslational regulation in the poorly differentiated one. The data concerning normal rat tissues confirm previous results on the rigorous genetic control of CuZnSOD under physiological conditions [31], a strict parallelism existing between transcripts and activities. Again, however, we observed a dissociation of these values from the metal ion content. It has to be noticed that the two tissues with the highest CuZnSOD activities, i.e. liver and kidney, are tissues with high metabolic activities. The first is characterized by a well developed endoplasmic reticulum where numerous detoxification reactions occur and by a high level of cytoplasmic redox reactions, both associated with ROS generation. The second is also characterized by an intense requirement of energy production and therefore by a high level of oxygen consumption.

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