Iron modulation of LPS-induced manganese superoxide dismutase gene expression in rat tissues

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Abstract Mitochondrial superoxide dismutase (MnSOD) is usually diminished in cancer cells. We observed that in vivo treatment with LPS produces a strong increase of MnSOD mRNA levels and a weak induction of an inactive protein in rat hepatocarcinomas. In normal liver iron deficiency, obtained with desferrioxamine administration, produces a decrease in the MnSOD induction by LPS, indicating that such induction could depend on tissue iron content. However, no change in MnSOD mRNA has been observed in iron-overloaded tumor tissue. Thus, iron is possibly involved in the transcriptional regulation of the protein, in combination with some other unknown factor that appears to be deficient in tumor cells.

Key words: Superoxide dismutase; Gene expression; Rat hepatocarcinoma; Liver; Lipopolysaccharide

1. Introduction

Reactive oxygen species (ROS) such as H_2O_2 and $O_2^{\bullet}$ are recognized to be involved in both the initiation and promotion phases of carcinogenesis [1].

The superoxide dismutases (SODs) are metalloproteins found both in prokaryotic and eukaryotic cells and their function is to scavenge $O_2^{\bullet}$ radicals. In mammalian tissues three major types of SOD exist: the copper-zinc containing SOD (CuZnSOD) localized in the cytosol, the extracellular SOD (EC-SOD) localized in extracellular fluids and the manganese containing SOD (MnSOD) found in the mitochondrial matrix [2–4]. It has been reported that MnSOD activity is decreased in transplantable tumors in vivo and tumor cell lines in vitro [5,6] but it is still uncertain whether the decrease of MnSOD in tumor tissues is related to a change in the dosage of MnSOD gene.

Previous works reported that the deficiency of this metalloenzyme would depend on the decrease of gene dosage. Indeed, a close association between the diminution of MnSOD activity, immunoreactive protein, mRNA and deletion of chromosome segment 6q21, where the gene is mapped, has been observed in several tumor cell lines [7]. Moreover, since the introduction of human chromosome 6 in human melanoma cells can suppress the malignant phenotype [8] and in human breast cells the overexpression of MnSOD has been reported to revert the malignant phenotype [9], MnSOD gene has been proposed to act as a tumor-suppressor gene [10].

On the other hand, in contrast to these observations, it was found that in transformed human lung fibroblasts (SV-40/WI-38), which are characterized by a significant degree of deletion of segment 6q21, there is no change in the dosage of the MnSOD gene or defect in the primary structure of the protein, although reduced levels of MnSOD activity, immunoreactive protein and mRNA are present [11].

Changes in MnSOD expression have been observed both in vitro and in vivo in normal eukaryotic cells and in tumor tissues, in response to a variety of physical or chemical agents [12–15].

Previously, in our laboratory, we obtained evidence in favor of a down-regulation of MnSOD at the (pre-)transcriptional level in poorly differentiated rat hepatocarcinomas where iron and manganese ions have been found to be markedly reduced [16,17].

In this study we examined the effects of the bacterial endotoxin lipopolysaccharide in tumor tissues in vivo. We used as experimental models the highly malignant rat hepatocarcinoma Morris 3924A (HCC) and liver from healthy rats. Treatment consisted of administration of LPS, a recognized mediator of the inflammatory response, able to stimulate TNF-α production in vivo.

Since we proposed that iron deficiency could be a factor responsible for the down-regulation of MnSOD in cancer cells [17], we thought to produce iron depletion in rat liver and iron overload in HCC tissue, in order to study the regulation of MnSOD after LPS induction and its relationship with the iron tissue content.

2. Materials and methods

2.1. Animals, tumors and treatments

Morris 3924A hepatomas (poorly differentiated and fast-growing) were propagated by subcutaneous transplantation in ACI/T inbred rats [6]. The tumors were utilized after 3–4 weeks of growth. Livers from normal inbred ACI/T were used as control.

Rats were treated intraperitoneally with 5, 10, 20, or 22.5 mg LPS/kg b.w. and killed after 18 h (LPS from *Escherichia coli* 0111:B4, Sigma). Desferrioxamine (Desferal, from Ciba-Geigy S.A.) was administered intramuscularly at a dose of 200 mg/kg b.w./day for 3 days before LPS treatment. Iron (Ferlixit from Rhone-Poulenc Rorer) was administered intravenously at a dose of 20 mg/kg b.w./day for 3 days before LPS treatment.

2.2. mRNA isolation and Northern blot analysis

Total RNA was isolated according to the method of Chirgwin et al. [18]. RNA concentrations were determined spectrophotometrically by absorbance at 260 nm. Samples of denatured RNA were size-fractionated by formaldehyde-agarose (1.5%) gel electrophoresis and capillary transferred to nylon membranes. Blots were prehybridized for 5 h at 42°C and hybridized to CuZnSOD and MnSOD cDNA probe overnight at the same temperature. The cDNA probe was radiolabelled with [α-^{32}P]dCTP using a Multiprime DNA labelling system (Amersham). Autoradiograms were obtained at 70°C using Kodak

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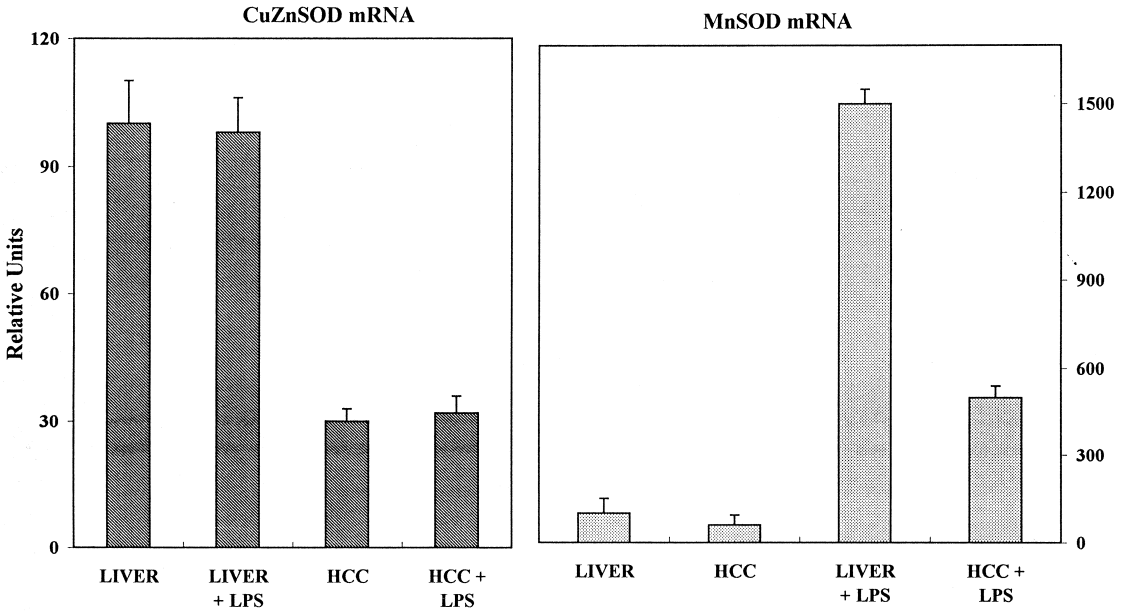

Fig. 1. Levels of CuZnSOD and MnSOD mRNA in liver and hepatocarcinoma (HCC) treated with 10 mg/kg b.w. LPS. Values are means ±S.E. from 3–4 experiments. MnSOD mRNA levels correspond to the sum of the intensities of the two major hybridizing bands (1080 and 850 nt) normalized to 100% for control liver.

XAR film. The intensity of autoradiograms was scanned with a transmission densitometer.

2.3. Superoxide dismutase activities

The tissues were excised and rinsed in saline to remove contaminating blood and then homogenized on ice in 0.05 M potassium phosphate, 0.1 mM EDTA, pH 7.4 for 2 min at full speed with a Polytron homogenizer. Proteins were estimated with the biuret method. MnSOD activity was assayed on 48 h-dialyzed homogenate of tumor and normal tissue specimens by the method of inhibition of hematoxylin autoxidation to hematein [19], monitored at 560 nm, at pH 7.5 and 25°C, using a standard curve obtained with purified bovine blood enzyme. MnSOD was measured in the presence of 1.5 mM Na cyanide.

2.4. Western blot analysis

SDS-polyacrylamide gel electrophoresis was performed on a 12.5% gel, with a 3% stacking gel. Gels were each 2 mm thick. 30 μg of total protein/well was loaded for the detection of MnSOD. After SDS-PAGE, proteins were horizontally electroluted into nitrocellulose paper with a transfer time of 1 h and a voltage of 100 mV. Immunoblot detection of the enzyme was performed with the monoclonal antibody directed against the rat MnSOD protein [20].

2.5. Ion content measurements

Metal concentrations were determined by atomic absorption using a Perkin-Elmer 272 spectrophotometer utilizing thin slices of fresh tissues dried overnight at 100°C and digested with 1 N nitric acid.

3. Results

3.1. Effects of LPS treatment on MnSOD in rat hepatocarcinomas and livers

In order to elucidate the molecular mechanisms responsible for the MnSOD alteration in cancer tissues we investigated in HCC and in rat hepatocytes the in vivo effects of LPS on MnSOD expression. In this experimental model we evaluated the activity, the immunoreactive protein and the mRNA of MnSOD.

A significant increase (about 15 times compared to the untreated tissue) in mRNA levels for MnSOD was observed in the liver of rats treated with LPS (10 mg/kg b.w. 18 h after treatment). In the tumor the MnSOD mRNA was also increased by LPS treatment, but it was only 7 times higher

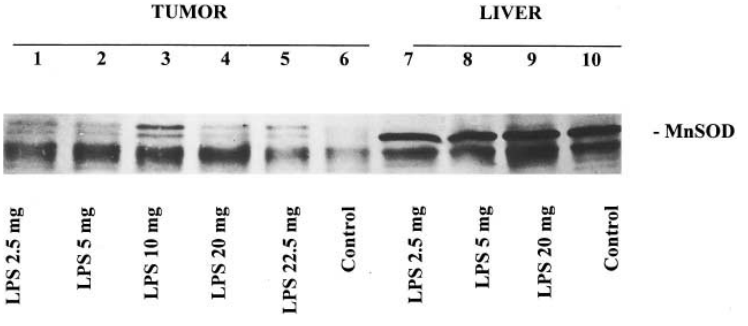

Fig. 2. Western blot of MnSOD in liver and HCC of rats treated with different doses of LPS: HCC of rats treated with 2.5, 5, 10 or 22 mg/kg b.w. LPS (lanes 1–5 respectively); HCC of control rat (lane 6); liver of rats treated with 2.5, 5 or 20 mg/kg b.w. LPS (lanes 7–9 respectively); liver of control rats (lane 10). The major MnSOD band represents a 24 kDa protein.

Table 1
Superoxide dismutase activities in hepatocarcinoma (HCC) and liver of control and treated rats

Treatment	MnSOD (μg/g wet weight)	CuZnSOD (μg/g wet weight)
Control liver	206.0 ± 31.7 (7)	355.5 ± 23.2 (7)
Liver+LPS	246.0 ± 24.6 (5)	354.1 ± 19.3 (5)
Liver+desferrioxamine	173.3 ± 21.3 (5)	354.6 ± 32.6 (5)
Liver+desferrioxamine+LPS	215.6 ± 35.0 (5)	330.1 ± 61.6 (5)
Control HCC	22.7 ± 4.2 (5)	23.0 ± 1.2 (5)
HCC+LPS	21.0 ± 3.3 (5)	25.4 ± 0.05 (5)
HCC+iron+LPS	19.3 ± 2.5 (5)	22.9 ± 3.9 (5)

Values are means ± S.E. Numbers in parentheses indicate the number of livers or hepatocarcinomas.

than that observed in control HCC. The stimulation with LPS did not affect the level of CuZnSOD mRNA, demonstrating a selective induction for the mitochondrial enzyme (Fig. 1).

The MnSOD protein contents of livers and tumors of rats treated with different doses of LPS were estimated by immunological staining in Western blots. Fig. 2 shows that in hepatocarcinomas from treated rats different doses of endotoxin (lanes 1–5) induced the 24 kDa band of MnSOD, which is nearly absent in the control HCC (lane 6), and a 25 kDa band, presumably corresponding to the cytosolic precursor of the protein [24]. The enzymatic activity, on the contrary, was not increased (Table 1). In rat HCC LPS also produced an increase of peptides of lower molecular weight (22 kDa band, recognized by the monoclonal antibody against rat MnSOD), which could likely be degradation products of MnSOD protein.

In treated rat livers (Fig. 2), only the 22 kDa band (lane 9) was induced by LPS compared to the control (lane 10) and only by a high dose of LPS (20 mg/kg b.w.). Lower doses of LPS (2.5 and 5 mg/kg b.w.) did not affect MnSOD content (lane 7, 8). In rat liver, LPS did not produce any increase of the 24 kDa band (Fig. 2).

The activity of the mitochondrial enzyme was slightly increased by LPS (10 mg/kg b.w.) in rat liver and unchanged in tumor tissues (Table 1).

3.2. *Effect of iron and desferrioxamine treatments on MnSOD in rat hepatocarcinomas and in rat liver*

In order to determine if iron plays a role in the regulation of gene expression of MnSOD in normal and tumor cells, we modulated iron content in rat liver and HCC. We treated healthy ACI/T rats with the iron chelator desferrioxamine (DESF) in order to decrease the iron tissue content and supplemented tumor-bearing rats with iron to increase the metal content.

The amount of iron in hepatocarcinoma cells is about 40% of that in normal hepatocytes (Fig. 3). A 3 day administration of the iron chelator DESF caused a 20% reduction of iron content in rat liver (Fig. 3) and this condition strongly reduced the induction of MnSOD mRNA obtained in response to LPS (Fig. 4). This finding suggests that iron can be involved in MnSOD regulation. On the other hand, the hepatic levels of MnSOD mRNA were almost unchanged after DESF administration without LPS stimulation (Fig. 4). Table 1 shows that MnSOD activity was almost the same in the livers of animals treated with DESF or with DESF plus LPS; more-

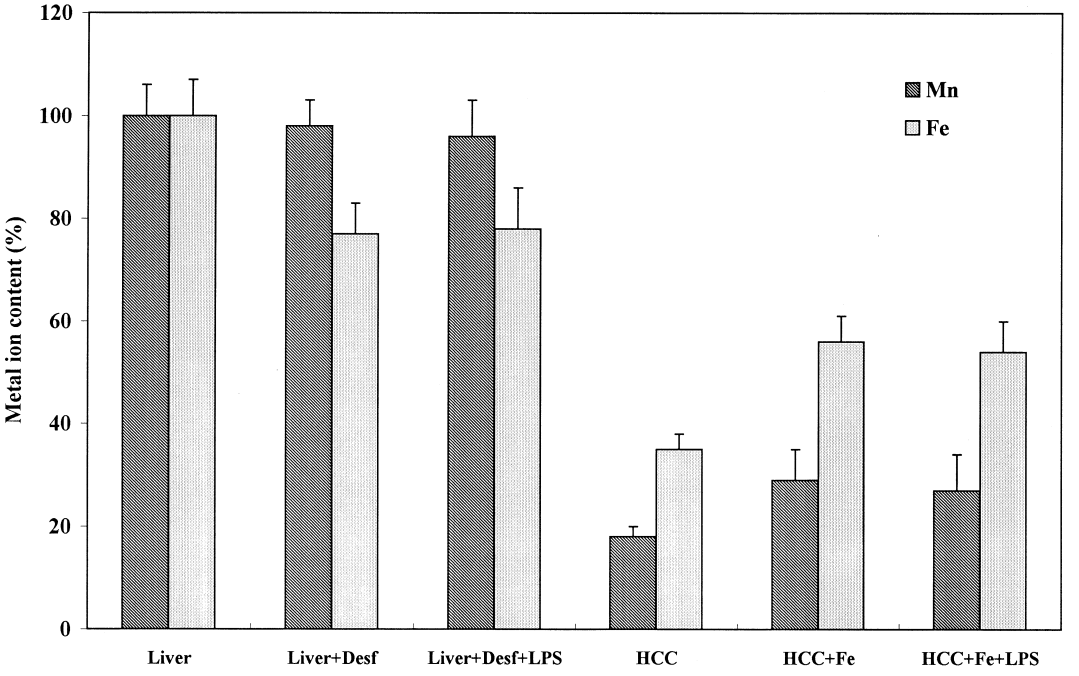

Fig. 3. Iron and manganese content in control liver and liver from rats treated with desferrioxamine (DESF), and DESF+LPS, in control HCC, HCC after iron overloading (HCC+Fe) and HCC with iron overloading plus LPS (HCC+Fe+LPS). Results are expressed as % of control liver (7 μg Mn and 350 μg Fe/g dry weight). Values are means ± S.E. from 6–7 rats.

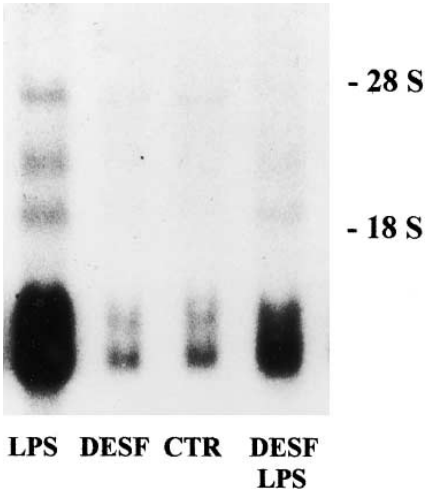

Fig. 4. Northern blot hybridization of MnSOD cDNA probe with total RNA isolated from rat liver treated with 10 mg/kg b.w. LPS, with the iron chelator DESF, control liver and liver pretreated with DESF plus LPS (lanes 1–4 respectively). The three minor bands were estimated to be about 4100, 3000 and 2100 nt and the two major band 1080 and 850 nt.

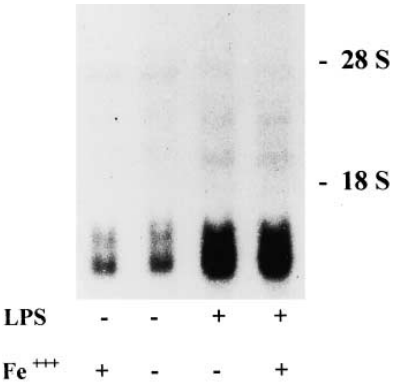

Fig. 5. Northern blot hybridization of MnSOD cDNA probe with 11 μg of total RNA isolated from HCC with iron overload, control HCC, HCC treated with LPS and HCC with iron plus LPS (lanes 1–4 respectively).

over, the immunoreactive protein was unchanged (data not shown).

Iron supplementation in rats bearing HCC produced a 50% increase of this metal in tumor tissue. The metal content obtained by iron overloading in tumor cells is still lower than in normal hepatocytes and it was not affected by LPS administration (Fig. 3). In the above experiments a modest increase of manganese content was also observed. This can be presumably attributed to the increase of saturation by Fe loading of plasma transferrin, a common carrier of the two ions [21,22].

The effect of LPS treatment on MnSOD mRNA was the same in the iron supplemented and the non-supplemented tumor tissues (Fig. 5). Moreover, in rat HCC we observed a decrease of the immunoreactive protein after iron treatment (Fig. 6).

4. Discussion

Tumor cells are known to differ biochemically in many ways from normal cells. Among the differences, a lack of MnSOD activity in vivo and in vitro has been reported (see [23] for review). The Mn-containing SOD is an inducible enzyme [2] and ROS are one of the major factors that regulate MnSOD gene expression [24]. We have used a rat hepatocarcinoma with a low degree of differentiation and control normal liver as models to study the mechanisms involved in the control of MnSOD gene expression in tumor cells in vivo.

In this study, we demonstrated that it is possible to induce the expression of MnSOD in vivo in the tumor using LPS. This bacterial endotoxin can increase mitochondrial superoxide dismutase expression in cell lines and in normal cells in vitro [13] and in stomach of rat in vivo [14] with a mechanism that is thought to stem from a response to the intracellular generation of cytokines, such as TNFα, and oxidants. The MnSOD induction by LPS may derive from cytokine involvement or from direct activation of the enzyme as part of the initial phase of the acute inflammatory response [25]. Moreover, LPS treatment appears to up-regulate the transcription factor AP-1 [26], one of the possible candidates involved in MnSOD gene expression.

In this work we obtained a strong MnSOD mRNA increase in both normal and tumor liver tissue following LPS treatment. In addition, we observed that the extent of the induction is less pronounced in tumor tissue than in normal tissue. MnSOD activity was also increased, albeit at a low level, in

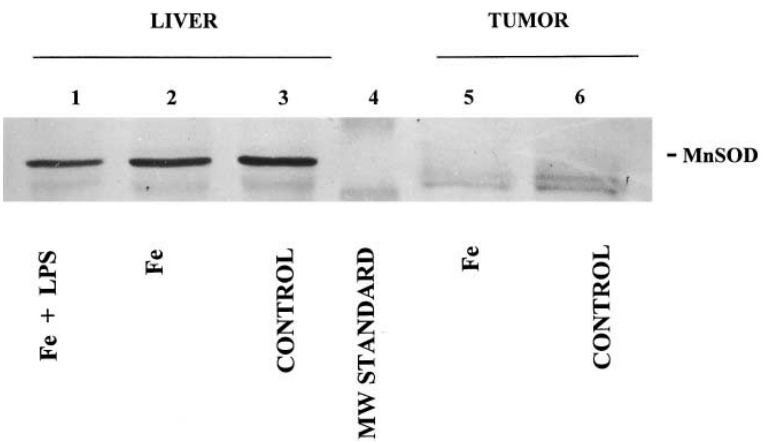

Fig. 6. Western blot analysis of HCC (lane 6) and liver (lane 3) of control rats, iron treated rat liver (lane 2), iron+LPS treated rat liver (lane 1) and HCC of iron treated rat (lane 5). Lane 4, molecular weight standard. The major MnSOD band represents a 24 kDa protein.

normal liver. In contrast, no significant increase of enzymatic activity was observed in the tumor, in spite of MnSOD mRNA and immunoprotein up-regulation.

In accordance with the observed increase of degradation products in LPS treated tumor tissue, previous investigations reported that LPS induces oxidative damage mediated by increased production of ROS and NO• [27,28], with an increase in lipid peroxidation and an alteration of the Ca^{2+} permeability of hepatocyte plasma membranes [29]. ROS are thought also to induce the transcription of early genes such as c-*fos*, c-*jun*, c-*myc* [30] or to activate directly DNA-binding proteins and transcription factors such as NF-kB and AP-1 [31].

In order to establish whether iron is involved in the regulation of MnSOD, we tried to mimic in rat liver by desferrioxamine treatment a condition of iron deficiency similar to that found in rat HCC [17]. In such experiments we observed a strong diminution of inducibility of the enzyme by LPS, supporting the above mentioned hypothesis. Nevertheless, the lack of induction of MnSOD mRNA in the iron overloading condition demonstrates that the metal alone is unable to up-regulate the enzyme. Altogether, the data reported suggest that induction of MnSOD by LPS depends partially on the iron tissue content and on factor(s) which are supposed to be lacking in highly malignant tumor cells.

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