In vivo Effect of Free Radical Scavenger Hepatoprotective Agents on Superoxide Dismutase (SOD) Activity in Patients

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The in vivo effects of two hepatoprotective antioxidants (silymarin, and 4-amino-5-imidazole-carboxamide-phosphate) on the expression and activity of superoxide dismutase (SOD) enzyme were studied in erythrocytes from patients with alcoholic cirrhosis. In vivo treatment with any of the drugs markedly increased the SOD expression of lymphocytes as measured by flow-cytometry followed by staining with monoclonal anti-Cu, Zn-SOD-antibody and FITC-conjugated anti-mouse Ig, as well as erythrocyte and lymphocyte SOD activities. The data indirectly suggest that antioxidant activity might be one of the important factors in the hepatoprotective action of these agents.

(Key Words: silymarin, Aica-P, superoxid-dismutase, antioxidants, hepatoprotection)

INTRODUCTION
Since the free-radical mechanism is supposed to play a major role in the pathogenesis of liver diseases (21, 22, 23, 24), antioxidant activity is one of the important mechanisms of action of potent hepatoprotective agents. As the natural flavonoid-type drug silymarin (Legalon®, MADAUS, FRG) and the imidazole derivative 4-amino-5-imidazole-carboxamide-phosphate (Aica-P, Chinoin-138, Chinoi, Hungary) belong to the group of free-radical scavenger agents (2, 4, 8, 11), we studied their effects on one of the most important antioxidant enzymes, superoxide dismutase (SOD, E.C.1.15.1.1) in lymphocytes and erythrocytes of patients with alcoholic cirrhosis and these of healthy controls. In our former studies we demonstrated decreased SOD activity and expression in erythrocytes and lymphocytes obtained from patients with chronic alcoholic liver disease (10, 16).

PATIENTS, MATERIALS AND METHODS
Thirty patients with chronic alcoholic liver disease (21 men and 9 women, mean age 44.9 yr, histological diagnosis: micronodular cirrhosis) were involved in the study. The silymarin group (daily 3 × 140 mg Legalon per os for one month) consisted of 8 men and 2 women, mean age was 46.3 yr. In the Aica-P group, 6 men and 4 women (mean age 43.4 yr) were treated with 3 × 200 mg Aica-P tablets per os for one month. A placebo group contained 7 men and 3 women, mean age was 44.4 yr.

The SOD activity of erythrocytes was determined by the original method of Misra and Fridovich (15). The method is based on the spontaneous autooxidation of epinephrine to adrenochrome at pH 10.2 in the presence of air. The inhibition of this process depends on the amount of SOD. Results are given in units, one unit referring to the amount of enzyme causing a 50% inhibition of autooxidation/min. Spectrophotometric measurements were performed with a Spectronom 204 equipment at 37°C and 480 nm. Erythrocyte lysates from patients were prepared by adding distilled water to 500 μl packed erythrocytes previously washed with isotonic saline. Haemoglobin was precipi-
tated with a chloroform ethanol mixture. The pale yellow supernatant obtained after centrifugation was used for estimating the specific activity of SOD.

Lymphocytes were separated from heparinized venous blood on Ficoll-Uromiro gradient (3). Phagocytic cells were removed by carbonyl iron treatment. Viability as judged by trypan blue test was greater than 95%, monocyte contamination was less than 2%. Cells were suspended in phosphate-buffered saline (PBS). Lymphocytes membranes were disrupted by sonication in icecool PBS. After centrifugation, enzyme analysis was performed in the supernatant. All data were expressed as units of total SOD/ml sample.

For the flow-cytometric evaluation of SOD-expression in lymphocytes, separated peripheral blood mononuclear cells were depleted of phagocytic cells by carbonyl iron treatment. 10^7 lymphocytes were incubated at 4°C with 5 μl monoclonal anticalf-Cu, Zn-SOD-antibody (17), diluted to 1:10 in PBS. Following washing in PBS, cells were stained for 30 min at 4°C with 5 μl rabbit anti-mouse Ig, conjugated with FITC (DAKO). After three washings in cold PBS, lymphocyte counts were adjusted to 3 million cells/ml and fluorescence patterns of cell suspensions were evaluated using a Tc 4800 A Cytofluorograph (Bio Physics system, Inc. USA).

In the in vivo treatment studies, patients were tested immediately before and after the treatment period.

Statistical analysis was performed by Student's paired t-test.

RESULTS

Total SOD activities of the erythrocytes and lymphocytes of cirrhotic patients and of healthy control subjects are summarized in Table 1. Patients with micronodular cirrhosis had significantly lower erythrocyte and lymphocyte SOD activity than the mean values for the healthy control group (Table 1). Essentially the same observation was made by cytofluorimetric analysis of the SOD expression of lymphocytes. A typical fluorescence distribution histogram is shown in Fig. 1.

One month in vivo treatment with silymarin significantly increased SOD activity of erythrocytes and lymphocytes of the patients (Fig. 2).

The originally low cytofluorimetric SOD expression of the patients' lymphocytes was also restored (Fig. 5).

In vivo treatment with Aica-P significantly increased the SOD activity of erythrocytes and lymphocytes of the patients (Fig. 4).
The same was true for the SOD expression of the patients' lymphocytes (Fig. 5).

DISCUSSION

The successful treatment of alcoholic cirrhosis has not yet been solved. Recently, the naturally occurring flavonoid silymarin has been widely used for this purpose (1, 14, 20, 25). Our previous findings concerning the therapeutic value of the imidazole derivative 4-Amino-5-imidazole-karboxamid have also been promising in chronic liver disease (9). The antioxidant effect, supposed to be one of the important factors in the mechanism of action of potent hepatoprotective drugs (19), as well as previous data suggesting the free-radical scavenger activity of the above-mentioned agents (2, 4, 8, 11) prompted us to investigate their in vivo effects on the expression and activity of SOD enzyme. Cu, Zn-SOD has been considered to be the central component in the intracellular defence mechanism of human cells against oxygen stress (18).

We postulated that the changes in SOD activity and expression following in vivo therapy may indirectly indicate their capacity to exert

| Table 1 Erythrocyte and lymphocyte SOD activity of cirrhotic patients and healthy controls |
|---------------------------------------------|-------------------|
| patients | Erythrocyte | Lymphocyte |
| n = 20 | 57.3 ± 11.2xx | 25.2 ± 7.9xx |
| healthy controls | 154.7 ± 18.9 | 103.6 ± 15.8 |

results in U/ml, mean ± SEM
xxp < 0.001 vs. control values
Fig. 1  SOD expression of lymphocytes from healthy and cirrhotic subjects.
LC: lymphocyte count, F: fluorescence

Fig. 2  Effect of treatment with silymarin on the SOD activity of erythrocytes and lymphocytes from cirrhotic patients.
Fig. 3  Effect of treatment with silimarin on the SOD expression of lymphocytes from cirrhotic patients.

Fig. 4  Effect of therapy with Aica-P on the SOD activity of erythrocytes and lymphocytes from cirrhotic patients.
free-radical scavenger effect.

In our experiments in vivo treatment with silymarin and Aica-P restored the originally low SOD activity and expression of the patients' erythrocytes and lymphocytes. It is noteworthy that, although cytofluorimetric expression (i.e. specific immunofluorescence following staining with monoclonal anti-SOD) and functional activity (i.e. inhibition of epinephrine—adrenochrome autooxidation) of the SOD enzyme may reflect different aspects of antioxidant capacity, the changes in these parameters were always similar. Decreased SOD expression and SOD activity may reflect an increased enzyme consumption as a consequence of enhanced production of free radicals. This assumption is in accordance with the observation of Fridovich (12). Previous findings concerning erythrocyte SOD activity of patients with alcoholic liver disease are rather controversial. Emerit et al. (6) found increased erythrocyte Cu, Zn-SOD activity in alcoholic patients. Our results are in good accordance with the findings of Goebel et al. (13).

The fact that in vivo treatment with these hepatoprotective agents increased SOD activity and expression may indirectly suggest the in vivo antioxidant effect of these drugs.

Further studies are under way in our laboratory to elucidate the possible significance of the decreased SOD expression and activity in patients with alcoholic cirrhosis and their partial restoration following in vivo treatment with hepatoprotective antioxidant agents.

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REFERENCES


