Decrease of superoxide dismutase and glutathione peroxidase in liver of rats treated with hypolipidemic drugs

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1. INTRODUCTION

Clofibrate and other hypolipidemic drugs increase the number of hepatic peroxisomes and cause a corresponding increase of several peroxisomal enzyme activities [1,2]. This effect regards H_2O_2-generating reactions (most typically, palmitoyl-CoA oxidase activity) and catalase, which is able to destroy most of the H_2O_2 formed within the organelle. It is reasonable to expect that the net flux of H_2O_2 outside the organelle should be enhanced under hypolipidemic treatment in view of the fact that significant H_2O_2 diffusion is observed in peroxisomes of untreated animals [3,4] and the increase of palmitoyl-CoA oxidase activity induced by clofibrate feeding is much higher than that of catalase [1]. Moreover, hypolipidemic agents cause proliferation of hepatic smooth endoplasmic reticulum as well, and this produces a marked increase of cytochrome P450 [5], which is likely to be a major source of O_2^- and H_2O_2 in the cell as suggested by results obtained with subcellular preparations [6,7]. It seemed therefore interesting to investigate whether activities of cytoplasmic enzymes acting as a defense against 'oxygen radicals' (i.e., superoxide dismutase and glutathione peroxidase) are affected by feeding hypolipidemic drugs. The results reported here show that both enzymes are significantly decreased in rat liver during treatment with clofibrate and procetofene, another hypolipidemic agent [8]. This effect is associated with an increased susceptibility of the tissue to enhanced peroxidative risk, as detected by malonyl dialdehyde formation under conditions known to give rise to peroxidizing 'oxygen radicals' [9].

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2. MATERIALS AND METHODS

2.1. *Materials*

Purest grade chemicals were used: *t*-butylhydroperoxide was obtained from Merck-Schuchardt; GSH, GSSG reductase, NADPH, and purest grade catalase (~65 000 U/mg) were purchased from Boehringer; bovine serum albumin (BSA) was obtained from Sigma Chemical Co.; superoxide dismutase (SOD) was purified from bovine erythrocytes as in [10]; ADP and 1,1,3,3-tetraethoxypropane (TEP) from Fluka, ethyl α-(*p*-chlorophenoxy)isobutyrate (clofibrate) and isopropyl-α-(4-*p*-chlorobenzoyl phenoxy)iso-butyrate (procetofene) were obtained from CFM, Milan.

2.2. *Animals and treatment*

Male (140–160 g) and female (120–140 g) Wistar rats were treated for 30 days with daily per os doses of 300 mg clofibrate/kg and 100 mg procetofene/kg. Such doses are currently used for animal experimentation with hypolipidemic drugs [8,11]. After killing, livers were exhaustively perfused with saline to remove blood, and then homogenized (1:6, w/v) with 5 mM phosphate buffer (pH 7.4) containing 0.2 mM EDTA. Before assaying for the enzyme activities, homogenates were sonicated for 5 min, in 30 s bursts, in ice bath. The microsomal and cytosolic fractions were prepared as in [12].

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2.3. *Assay methods*

Protein was determined as in [13] with bovine serum albumin as a standard. SOD was determined by a polarographic method [14] and its activity was expressed as μg enzyme/mg protein, with reference to a sample of purified bovine enzyme. Glutathione peroxidase (GSH-Px) activity was assayed with a spectrophotometric method using *t*-butyl-hydroperoxide as substrate [15]. Aspartate aminotransferase (AAT) activity was measured as in [16]. Both these enzyme activities are expressed as units (M substrate transformed . min^{-1} . mg $protein^{-1}$). Aldehyde oxidase activity was assayed with pyridoxal as substrate by measuring at alkaline pH the fluorescence of the product pyridoxic acid in its lactone form [17]; the activity is expressed as fluorescence intensity (F)/mg protein. Catalase and urate oxidase activities were measured by UV methods and expressed in the units [18,19].

Susceptibility to lipid peroxidation was detected by malonyl dialdehyde (MDA) formation after oxidative challenge of liver homogenates or microsomal fractions with ADP–Fe^{3+} in the presence of NADPH [9]. MDA was measured by a colorimetric procedure involving reaction with thiobarbituric acid [20]; the results are expressed as nmol . MDA^{-1} . mg $protein^{-1}$ using TEP as an external standard.

3. RESULTS

Table 1 shows the effects of clofibrate and procetofene on several enzyme activities of rat liver. The increase of catalase, which is known to be much higher in male animals [21], has to be confronted with the unaltered values of AAT and urate oxidase and the significant lower activities of GSH-Px and SOD. In the case of procetofene the decrease of the 2 latter enzyme activities is much more evident in male than in female animals. Table 1 also reports

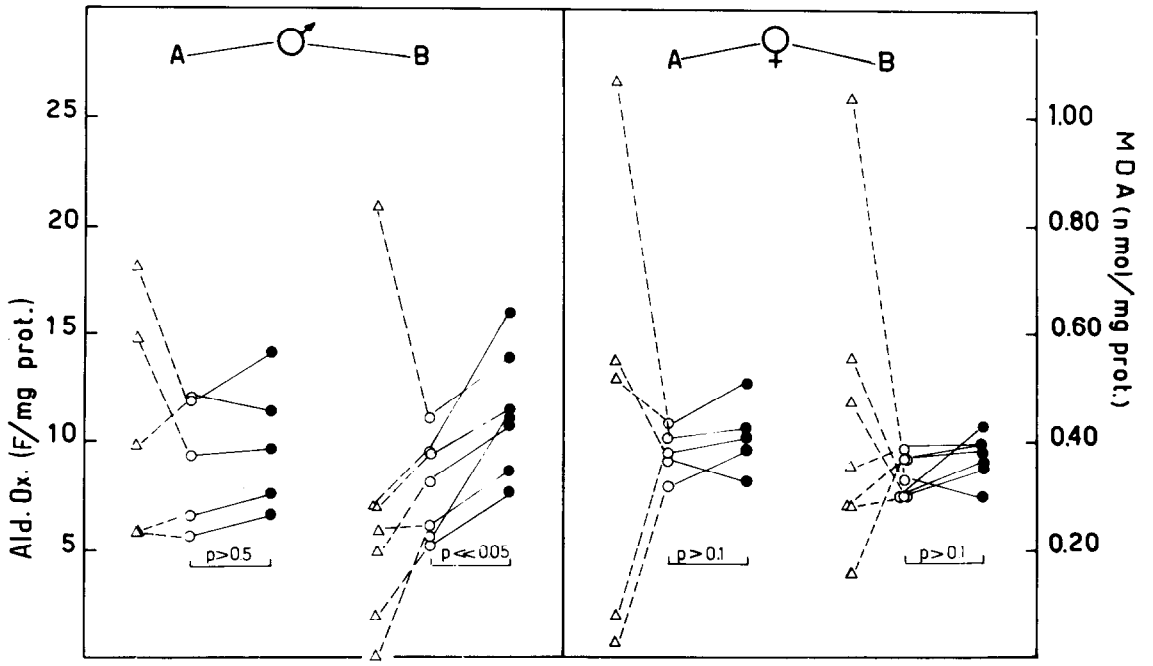

Fig.1. MDA production by liver homogenates of rats fed with procetofene upon treatment with ADP–Fe^{3+} in the presence of NADPH. Total liver protein (70 mg) was incubated for 2 h at 37°C in the presence of 4 mM ADP and 0.012 mM $FeCl_3$ with 0.4 mM NADPH as substrate, in 5 mM phosphate buffer (pH 7.4). The figure shows MDA production in the absence (○) and in the presence (●) of NADPH and ADP-Fe^{3+} by liver homogenates of male (♂) and female (♀) control (A) and procetofene-fed (B) rats. The activity of aldehyde oxidase (△) of the same liver homogenates is also shown. The data pertaining to the same sample are connected by dotted (ald.ox. *vs* basal MDA) and solid (basal *vs* induced MDA) lines.

Table 1

Enzyme activities in liver homogenates of rats fed with clofibrate or procetofene

Sex	Treatment	SOD (μg/mg protein)	p	GSH-Px (U/mg protein × 10^4)	p	GSH-Px / SOD	AAT (U/mg protein × 10^4)	Catalase (U/mg protein)	Urate oxidase (U/mg protein)
Male	Control	2.53 ± 0.32		2.53 ± 0.20		1.0	2.80 ± 0.10	10.87 ± 1.03	2.67 ± 0.19
	Clofibrate	1.98 ± 0.25	$\ll$0.001	2.00 ± 0.08	<0.001	1.0	2.85 ± 0.25	26.91 ± 1.34	2.96 ± 0.17
	Procetofene	1.85 ± 0.24	$\ll$0.001	2.04 ± 0.26	<0.001	1.1	2.90 ± 0.51	25.60 ± 0.64	2.80 ± 0.21
Female	Control	2.71 ± 0.35		4.69 ± 0.50		1.7	2.00 ± 0.10	8.60 ± 1.75	3.08 ± 0.25
	Clofibrate	1.96 ± 0.29	<0.01	2.85 ± 0.03	<0.001	1.5	2.15 ± 0.14	12.21 ± 1.56	3.21 ± 0.21
	Procetofene	2.38 ± 0.18	<0.05	3.56 ± 0.38	<0.001	1.5	2.29 ± 0.17	12.28 ± 1.25	2.93 ± 0.41

Data are expressed as mean values ± SD. Statistical evaluation were performed by Student's t-test: $p < 0.05$ = significant. Each group consists of 10 animals. Catalase values were the same after homogenization in the presence of Triton X-100 and treatment of the homogenate with ethanol to destroy any inactive complex II [26].

the values of the ratio between the GSH-Px and the SOD activities under the different conditions. This value is higher in female rats independently of treatment, the GSH-Px activity being sex-dependent. No effects of the drugs on GSH-Px and SOD activities were observed upon addition of 5 mM drug to the assay system, even after 7 h incubation at room temperature.

Fig.1 reports the results of MDA determinations in liver homogenates after incubation with ADP–Fe^{3+} in the presence of NADPH. MDA was found to form over the endogenous level only in treated male rats. Aldehyde oxidase activity, which could affect the level of aldehyde actually detected, was also determined in all samples. The variations measured were found to be very large and not related to MDA values. Therefore they are not to be considered causative of the amount of MDA formed.

The ADP–Fe^{3+}-dependent lipid peroxidation is usually studied with isolated microsomal fractions [9]. Therefore, the data in fig.1 cannot be used for any valid interpretation of the mechanism by which ADP–Fe^{3+} induces increased MDA production in male rats only. Isolated subcellular fractions were used to test the hypothesis that MDA production in these conditions can actually be related to antioxidant enzyme systems.

Table 2

ADP–Fe^{3+}-dependent MDA production by rat liver microsomes [9] under various conditions

Incubation mixture	MDA increase (%)
1. Microsomes (2.5 mg protein/ml	675
2. Microsomes + cytosolic fraction (8 mg protein/ml containing 0.6×10^{-6} M SOD, 25×10^{-4} U GSH-Px/ml and 20 U catalase/ml)	318
3. Microsomes + heat-treated cytosolic fraction (10 min in boiling water bath); residual enzyme activities: 0.02×10^{-6} M SOD; 0.26×10^{-4} U GSH-Px/ml; catalase, not detectable	533
4. Microsomes + BSA (8 mg/ml)	700
5. Microsomes + 500 U catalase/ml	250
6. Microsomes + 2×10^{-6} M SOD	785
7. Microsomes + 500 U calatase/ml + 2×10^{6} M SOD	197

[a] The total cell homogenate contains 3×10^{-6} M SOD, 88×10^{-4} U GSH-Px/ml and 400 U catalase/ ml

The results are expressed as % induced MDA, with respect to the endogenous level (i.e., in the absence of ADP–Fe^{3+})

Table 2 reports a series of experiments carried out to this aim. The results show that catalase at a concentration comparable to that of the total homogenate inhibits ADP–Fe^{3+}-dependent MDA production, while superoxide dismutase alone does not and even produces an increase of MDA production over the control value. However a mixture of SOD and catalase seems to have a higher inhibitory effect than catalase alone. Addition to microsomes of a cytosolic extract naturally equipped with SOD and GSH-Px and practically devoid of catalase had an inhibitory effect as well. Partial inactivation of SOD and GSH-Px by heat treatment of the cytosolic extract led to a roughly proportional loss of inhibition. The specificity of the effect of the cytosolic extract was supported by the lack of any inhibitory effect by BSA at a comparable protein concentration.

4. DISCUSSION

The decrease of GSH-Px and SOD activities in liver of rats treated with 2 hypolipidemic drugs is significant, not imputable to indiscriminate effects on cytoplasmic enzymes (AAT is not changed) and neither related to interference of the drugs with the assay system nor to inhibition of the enzyme activity. Such a repression of cytoplasmic enzymes is rather unexpected, as the effects of hypolipidemic drugs in [1,5] were restricted to membrane enzymes and associated with membrane proliferation. The nature of such a repression is obscure, however its clear sex-dependence (see table 1), analogous to that shown for peroxisomal enzymes [21], points to complex mechanisms, perhaps involving biosynthesis of the enzymes.

The relevance of the reported observations is 2-fold:

(i) They may offer an additional tool for studying the regulation of the cellular content of such important enzymes as those acting on active oxygen derivatives in the cytoplasm, i.e., SOD and GSH-Px;

(ii) They reinforce the idea that these 2 enzymes, and in particular GSH-Px, are key factors in the cell defense against deleterious effects of oxygen radicals.

The predominant role of GSH-Px in the cytoplasmic compartment was suggested [22] and implies that higher values of the GSH-Px/SOD ratio should reasonably produce a better control of the H_2O_2 flux in the cytoplasm.

In fact, SOD increases the rate of H_2O_2 production through O_2^- dismutation, and such an effect would result in harmful consequences if its activity is not balanced by proper amounts of GSH-Px. H_2O_2 is the most sable among partially reduced oxygen derivatives and is directly involved in all the molecular mechanisms of oxidative damage (peroxidation, Fenton reaction, Haber-Weiss reaction, etc.; see [23–25]. The results shown in fig.1 and table 2 are in line with this hypothesis. Under conditions of increased production of oxygen radicals from O_2^-, as those set by incubation of the tissue with NADPH and ADP–Fe^{3+} [9], more MDA, an established marker of enhanced lipid preoxidation, is formed in liver of treated male rats only. Livers of female rats, which seem to be insensitive in this regard, have a much higher GSH-Px content, and maintain rather high values of GSH-Px activity and GSH-Px/SOD ratio after treatment in spite of the decrease induced by the 2 hypolipidemic drugs. The relevance of protection against H_2O_2 present in total cell homogenate from ADP–Fe^{3+} is supported by the highly protective effect of catalase (table 2) added to a microsomal preparation, in contrast to the lack of any effect by SOD. The mixture of SOD and catalase inhibits more than catalase alone. These results confirm the above proposal: SOD itself would lead to an increased flux of peroxidizing H_2O_2 in the cell, however its concerted action with H_2O_2-removing enzymes improves the antioxidant capability of cells (perhaps *vs* $OH^{\cdot}$; see [23–25]. A further indication that the interpretation of the results of fig.1 is a valid one comes from the experiment where a cytosolic fraction was added to microsomes. The cytosolic extract showed specific protection (BSA, in fact, was ineffective) due to the presence of heat-labile enzymic factors. This protection is very likely to be due to the natural combination of GSH-Px and SOD activity: partial heat inactivation of the 2 enzymes is actually paralleled by loss of inhibition of MDA production.

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