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NORMOLIPIDAEMIC ACTIVITY OF LIPOSOMAL-ENCAPSULATED SUPEROXIDE DISMUTASE IN RATS

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To determine the regulatory effects of superoxide dismutase (SOD) on lipid metabolism a simple model of hyperlipidaemia induced by a hypercholesterolaemic (HCT) diet in rat was used. In animals fed a HCT diet, triglyceride (TG) were increased by 126%, total cholesterol (TCT) by 40%, very low density lipoprotein (VLDL) by 124% and the TCT/HDL ratio by 82%. The procedure would therefore appear to model some of the risk factors of atherogenesis.

In animals fed a hypercholesterolemic diet, liposomal Cu-SOD (200 μg/kg i.m. every two days; 1000 μg/kg i.m./day) decreased TG by 29 and 49%, TCT by 14 and 36%, TCT/HDL ratio by 32 and 60%, VLDL by 52 and 55% respectively and increased high density lipoprotein cholesterol (HDL-C) by 17 and 46% respectively.

The present experiments show therefore that the administration of liposomal SOD has a marked effect on lipid parameters (particularly TCT and TG) and might therefore reduce the atherogenic risk by increasing HDL and decreasing VLDL and cholesterol atherogenicity ratio (CAR).

KEY WORDS: Hypercholesterolemic diet, hyperlipidemia, liposomal copper-containing superoxide dismutase, normolipidemic activity, rats.

ABBREVIATIONS: CAR: cholesterol atherogeneicity ratio; Cu-SOD: copper-containing superoxide dismutase; HCT: hypercholesterolemic; HDL: high density lipoproteins; HDL-C: high density lipoproteins cholesterol; LDL: low density lipoproteins; LP: lipoproteins; MDA: malondialdehyde; NBT: Nitroblue tetrazolium; PL: phospholipids; SOD: superoxide dismutase; TBA: Thiobarbituric acid; TBARM: thiobarbituric acid-reactive material; TCT: total cholesterol; TG: triglycerides; VLDL: Very low density lipoproteins.

INTRODUCTION

Nearly half of all deaths in the industrialized world are attributed to atherosclerosis and its complications. Hyperlipidaemia (particularly hypercholesterolaemia) is an important risk factor in the onset of development of cardiovascular diseases.[1]

Atherosclerosis is currently considered to be dependent on several factors where an increase in blood lipids (cholesterol and LDL in particular) is associated with cellular lesions of vessel intima. Cholesterol and lipids in general are carried in the blood stream as lipoprotein complexes. Their role in such a pathological process has been

established by biochemical,[2] experimental,[3] clinical[4,5] and epidemiological studies.[6,7] Research into hypolipidemic drugs might therefore constitute a fruitful approach to the prevention and treatment of atherosclerosis. Superoxide anion ($O_2^{\cdot}$) and the free $OH^{\cdot}$ radical may be cytotoxic by their actions on proteins, cellular proteoglycans and mutagenic by their actions on DNA. Lipid peroxides, formed by the peroxidation of membrane unsaturated fatty acids (especially arachidonic acid) give via cyclo-oxygenase, besides cytotoxic endoperoxides, thromboxanes and prostacyclins, and via lipooxygenases, leukotrienes with chemotactic and cytotoxic properties.[8]

Furthermore, by a similar mechanism, this peroxidation transforms LDL, the main blood carrier of cholesterol into an abnormal form. This modified LDL is eliminated by means of a specific receptor, located on the macrophages and myocytes which are transformed into typical foam cells of atheroma.[9] Moreover clinical studies have shown that the lipidoperoxidation rate may provide an index of atherosclerosis severity.[10]

A recent study proved that probucol, known for its hypolipidemic effect, is able to inhibit oxidation of LDL by endothelial cells:[11] a possible antiatherogenic effect of antioxidant drugs must be investigated.

Furthermore, Michelson and co-workers have previously observed a significant hypolipidemic effect in animals treated with free bovine Cu-SOD and liposomal Cu-SOD: both blood TCT and TG were reduced, in contrast with controls.[12]

These authors showed in the same report that lipid peroxidation (measured by TBA test) significantly decreased, liposomal SOD being more efficient than the free enzyme. The reduction of lipid peroxide formation is a function of the dose of the enzyme administered. In various models these authors have also shown that longer lasting effects are obtained with the liposomal enzyme compared with the free one.

Thus, in our study, we wanted to test on this kind of pathogenesis a specific enzymatic antioxidant: SOD, an endogenous protein which protects cells against the damaging action of oxygen derived free radicals.

Considering the normolipidemic effects of antioxidant drugs and the specific anti-oxidant activity of SOD, the present experiments were aimed to demonstrate the activity of these agents on lipid metabolism.

For this purpose, rats were fed a hyperlipidemic diet and treated with bovine liposomal Cu-SOD, the most active form for treatment.

MATERIALS AND METHOD

1. Bovine liposomal Cu-SOD

The Cu-SOD was prepared according to Michelson *et al.*[13] from bovine blood collected on citrate. After lysis of the erythrocytes and precipitation of haemoglobin with ethanol-chloroform (0.25 v/0.15 v) followed by addition of K_2HPO_4 (300 g/l) and precipitation of proteins by acetone. The crude enzyme was dialyzed against an ethanolamine solution (25 mM) – acetic acid at pH = 8.0. This solution was purified by chromatofocussing on 50 ml PBE 94, eluting the column with polybuffer 96 in water (10% v/v) adjusted to pH 6.0 with acetic acid. Isoelectric focussing on gel gave a pI of 4.9. The specific enzymatic activity was approximately 3000–3300 unit/mg measured by using the riboflavin NBT method.

Cu-SOD were encapsulated in liposomes prepared in cationic form. Multilamellar vesicles with an average diameter of 350–400 nm were produced using a mixing of

L-α-dipalmitoyl lecithin, cholesterol and stearylamine (7:1:2) dissolved in chloroform.

This solution (2.5 ml) was evaporated to dryness under vacuum and 1 mg Cu-SOD in 4 mM phosphate buffer pH 7.2 (1 ml) was added. The mixture was warmed to 40°C and agitated for 5 minutes, then left at room temperature during 2–4 hours and at 15°C overnight.

Liposomes were then separated from uncapsulated Cu-SOD and components in excess, by centrifugation to 139 000 g for 2 hours at 4°C. The pellet was then lyophilized and stored at 4°C until administration.

2. Animals

Male adult gnotoxenic Wistar AF rats, 7 week old, weighing 167 ± 4.0 g were used (IFFA-CREDO FRANCE). The following conditions in animal house were kept constant: temperature: 22 ± 2°C; humidity: 50%; isolation from noise; artificial non-reversed light cycle: light 06:00–18:00 hr – darkness 18:00–06:00 hr.

3. Diets and treatments

5 groups of 10 animals were used:

1) Group A: 10 untreated rats were fed a standardized diet (U.A.R. 113 Villemoisson France) and water ad libitum;

2) Group B: 10 rats received a HCT diet over 30 days: (U.A.R. 214S: casein 14%, gelatin 6%, lard 33%, corn oil 2%, dextrose 36%, cholesterol 2%, sodium cholate 1.8%, mineral complex 4%, vitamins 1%, choline 0.2%);

3) Group C: 10 rats received a HCT diet over 30 days and were treated by liposomal SOD with 200 μg/1 ml/kg i.m. every two days;

4) Group D: 10 rats received a HCT diet over 30 days and were treated by liposomal SOD with 1000 μg/1 ml/kg i.m./day.

5) Group E: 10 rats received a HCT diet over 30 days and were treated by empty liposomes 1 ml/kg i.m./day.

All injections and manipulations were made at 09:00 a.m.

4. Assays

Rats were bled by cardiac puncture two hours after the last liposomal SOD injection (11:00 a.m.). The whole blood was collected without anticoagulant for serum lipid analysis.

Solid food was removed 12 hours before puncture.

a) Lipidogram: Lipoprotein electrophoresis was performed on cellulose acetate gel after coloration by red Ciba.[14,15]

b) PL, TG and TCT were determined using colorimetric and enzymatic reagents (Wako kits) described by Ikuta *et al.*,[16] Nishina[17] and Allain *et al.*[18]

c) HDL-C was separated using a heparin manganese precipitation procedure and assayed by a colorimetric and enzymatic method using Trinder's reaction.[19]

TABLE I
Effects of HCT diet and SOD treatment on body weight (expressed in grams) in control and treated rats

Group	m	± s.e.m.	Variance analysis		Comparisons between groups			
			Day 0					
A	172	3.76			B	C	D	E
B	166	4.48		A	ns	ns	ns	ns
C	165	5.01	$F = 0.87$ ns	B		ns	ns	ns
D	162	3.57		C			ns	ns
E	170	4.5		D				ns
			Day 15					
A	235	5.13			B	C	D	E
B	245	6.62		A	ns	ns	ns	ns
C	235	5.51	$F = 1.34$ ns	B		ns	ns	ns
D	239	5.25		C			ns	ns
E	250	5.73		D				ns
			Day 30					
A	283	6.18			B	C	D	E
B	317	8.56		A	**	ns	ns	**
C	304	7.01	$F = 4.31$ $p \leqslant 0.01$	B		ns	*	ns
D	287	6.32		C			ns	ns
E	311	7.47		D				*

Group A: 10 untreated rats fed a standardized diet.
Group B: 10 untreated rats fed a HCT diet.
Group C: 10 rats fed a HCT diet and treated for 30 days with 200 μg/kg every 2 days of SOD by i.m. injection.
Group D: 10 rats fed a HCT diet and treated for 30 days with 1000 μg/kg/day of SOD by i.m. injection.
Group E: 10 rats fed a HCT diet and treated for 30 days with empty liposomes 1 ml/kg/day by i.m. injection.
At each weight day the total significance was calculated by analysis of variance and Newman-Keuls method was performed to determine if differences occurred between groups.
ns = not statistically significant; * = $p \leqslant 0.05$; ** = $p \leqslant 0.01$.

5. *Animal weight*

To monitor growth rate the rats were weighed at days 1, 15, and 30 (Table I).

6. *Statistical analysis*

The data were analysed for statistical significance using one-way analysis of variance followed by individual comparisons between groups using the Newmann–Keuls method.[20]

RESULTS

1. *Weight gain*

There were no differences between the 5 groups at days 0 and 15. At day 30; the body weight gain of group B (10 untreated rats fed a HCT diet) and that of group E (10 rats fed a HCT diet and treated with empty liposomes) were greater than that of group A (10 untreated rats fed a standardized diet)($p \leqslant 0.01$) and that of group D (10 rats fed a HCT diet and treated with 1000 μg/kg/day)($p \leqslant 0.05$). We also observed that

the weight of the reference group A (10 untreated rats fed a standardized diet) and that of group D (10 rats fed a HCT diet and treated with 1000 μg/kg/day) increased similarly.

In spite of the HCT diet, rats treated at the highest dose of SOD grew like rats fed standardized diet.

2. Lipid variations

a) PL were similar in the different groups.

b) TG were increased in group B (10 untreated rats fed a HCT diet) and in group E (10 rats fed a HCT diet and treated with empty liposomes). SOD treatments normalized significantly the TG. TG of rats fed a HCT diet are decreased by 29% in group C (regimen of 200 μg/kg every two days) and by 49% in group D (regimen of 1000 μg/kg/day) in comparison with untreated rats fed a HCT diet (group B).

c) TCT was the highest in the rats fed a HCT diet and treated by empty liposomes. TCT level of treated groups was normalized by SOD treatments. TCT is also decreased by 14% and by 36% in treated groups in comparison with untreated rats fed a HCT diet.

d) The HDL-C: diet had no effect on HDL-C concentration. Only in the group D (regimen of 1000 μg/kg/day) was the HDL-C level significantly increased in comparison with that of untreated groups.

(e) The TCT/HDL-C ratio was considered as a CAR according to Castelli.[21] Values of this ratio were significantly different in the five groups. Treatment by liposomal SOD decreased this ratio even below the normal values for group D (regimen of 1000 μg/kg/day).

3. Lipoproteins

a) VLDL were greatly increased in untreated groups. Liposomal SOD reduced and normalized LP in treated groups.

b) LDL were similar in the different groups.

c) HDL were considerably decreased in untreated groups. Liposomal SOD increased and normalized HDL%.

All data are reported in Tables II and III.

DISCUSSION

Drugs which either decrease blood atherogenic lipids and lipoproteins (such as VLDL and LDL) or increase antiatherogenic HDL, are used in order to delay or eliminate the development of atherosclerosis.

Most of the tests currently used to study such drugs are based on the evaluation of the effects on circulating lipids in laboratory animals.[22]

In spite of the differences between human and rat lipidograms, animals are extensively used to assess a potential normolipidaemic activity on lipid metabolism. In this study, we used a simple model of hyperlipidaemia induced by a HCT diet in rat,

TABLE II
Effects of HCT diet and SOD treatment on plasma lipid levels in control and treated rats

Group	m	± s.e.m.	Variance analysis		Comparisons between groups			
			PHOSPHOLIPIDS mmoL/l					
A	1.61	0.11			B	C	D	E
B	1.54	0.15		A	ns	ns	ns	ns
C	1.41	0.09	$F = 1.99$ ns	B		ns	ns	ns
D	1.26	0.04		C			ns	ns
E	1.65	0.14		D				ns
			TRIGLYCERIDES mmoL/l					
A	0.91	0.09			B	C	D	E
B	2.04	0.11		A	**	**	ns	**
C	1.45	0.08	$F = 34$ $p \leqslant 0.001$	B	**	**	ns	
D	1.03	0.08		C			ns	**
E	2.31	0.15		D				**
			TOTAL CHOLESTEROL mmoL/l					
A	1.54	0.12		B	C	D	E	
B	2.16	0.19		A	*	ns	ns	*
C	1.87	0.11	$F = 5.59$ $p \leqslant 0.01$	B	ns	**	ns	
D	1.39	0.14		C			ns	ns
E	2.07	0.13		D				**
			HDL-C mmoL/l					
A	0.31	0.04			B	C	D	E
B	0.24	0.02		A	ns	ns	ns	ns
C	0.28	0.01	$F = 5.04$ $p \leqslant 0.05$	B		ns	*	ns
D	0.35	0.02		C			ns	ns
E	0.20	0.03		D				*
			TCT.HDL-C RATIO					
A	4.97	0.11		B	C	D	E	
B	9.81	0.08		A	**	ns	ns	**
C	6.68	0.22	$F = 270$ $p \leqslant 0.001$	B		ns	•	ns
D	3.97	0.07		C			ns	ns
E	10.35	0.28		D				•

Group A: 10 untreated rats fed a standardized diet.
Group B: 10 untreated rats fed a HCT diet.
Group C: 10 rats fed a HCT diet and treated for 30 days with 200 μg/kg every 2 days of SOD by i.m. injection.
Group D: 10 rats fed a HCT diet and treated for 30 days with 1000 μg/kg/day of SOD by i.m. injection.
Group E: 10 rats fed a HCT diet and treated for 30 days with empty liposomes 1 ml/kg/day by i.m. injection.
At each weight day the total significance was calculated by analysis of variance and Newman-Keuls method was performed to determine if differences occurred between groups.
ns = not statistically significant; * = $p \leqslant 0.05$, ** = $p \leqslant 0.01$.

because this hyperlipidaemia corresponds to the usual conditions of an atherogenic risk such as an associated increase in TG and TCT.

LDL are increased in most hyperlipidaemic models, but in the rat model we used, there was no increase in LDL rate whatever the treatment and the diet. On the other hand, in our model, VLDL were definitely increased (120%) using a hyperlipidaemic diet.

These conditions are similar to those used by Smith in the restricted ovulator hen model.[23] This fowl presents a hyperlipidaemia and develops atherosclerotic lesions.

TABLE III
Effects of HCT diet and SOD treatment on lipoprotein levels in control and treated rats

Group	m	± s.e.m.	Variance analysis	Comparisons between groups				
			VLDL (%)					
A	4.64	0.64			B	C	D	E
B	10.4	1.78		A	**	ns	ns	**
C	4.99	0.37	$F = 7.99$ $p \leqslant 0.001$	B	**	**	ns	
D	4.72	1.04		C		ns	**	
E	9.75	0.71		D		**		
			LDL (%)					
A	88.3	4.24			B	C	D	E
B	87.3	5.78		A	ns	ns	ns	ns
C	86.3	5.41	$F = 0.05$ ns	B		ns	ns	ns
D	89.7	6.42		C			ns	ns
E	87.7	5.88		D				ns
			HDL (%)					
A	7.04	0.66			B	C	D	E
B	2.41	1.08		A	**	ns	ns	**
C	6.49	0.84	$F = 8.22$ $p \leqslant 0.001$	B	**	**	ns	
D	7.91	0.88		C			ns	**
E	3.11	0.79		D			**	
			HDL/LDL RATIO					
A	0.079	0.005			B	C	D	E
B	0.028	0.006		A	**	ns	ns	**
C	0.075	0.005	$F = 27$ $p \leqslant 0.001$	B		**	**	ns
D	0.088	0.004		C			ns	**
E	0.035	0.006		D				**

Group A: 10 untreated rats fed a standardized diet.
Group B: 10 untreated rats fed a HCT diet.
Group C: 10 rats fed a HCT diet and treated for 30 days with 200 μg/kg every 2 days of SOD by i.m. injection.
Group D: 10 rats fed a HCT diet and treated for 30 days with 1000 μg/kg/day of SOD by i.m. injection.
Group E: 10 rats fed a HCT diet and treated for 30 days with empty liposomes 1 ml/kg/day by i.m. injection.
At each weight day the total significance was calculated by analysis of variance and Newman-Keuls method was performed to determine if differences occurred between groups.
ns = not statistically significant; * = $p \leqslant 0.05$; ** = $p \leqslant 0.01$.

The high level of blood lipids is considered responsible for the atherogenesis. Incriminated lipids are TG and TCT.

We also observed, besides an increase in TCT and TG, an increase in VLDL which in rabbit is probably a main factor of atherosclerosis.[11] It must be noted that, lipid peroxidation and free radicals play an important role in the atherogenic process according to Smith *et al.*[23] and Parthasarathy *et al.*[11] Thus it can naturally be assumed that the presence of oxygen-derived free radicals in excess and lipid peroxides in the arterial walls plays a role in the atheroma plaque formation.

Several studies have shown the importance of reactive forms of oxygen and lipid peroxides. It must be mentioned the tests performed in the albino New Zealand rabbit,[24] in which a HCT diet induced an increase of peroxidized lipids in plasma and aortic walls with an increase in glutathione disulfide. Loeper *et al.*[8] report on the increase of arachidonic acid in blood and within the intima in both man and rabbit,

while TBARM, which increased in plasma of both species, only increased in rabbit aortic wall.

The clinical tests of Stringer *et al.*[10] show a significant increase in lipid peroxides in 100 patients with angiographically-verified occlusive arterial disease in comparison with 75 control patients with no clinical evidence of atherosclerosis.

Some experimental tests have demonstrated the interest of using antioxidant drugs *in vivo* in order to reduce oxidative processes observed when LDL accumulate in the blood. We know that vitamin E is carried in plasma by lipoproteins and especially by LDL.[25] This fact seems to indicate that a diet supplemented with vitamin E might decrease lipid oxidation within these lipoproteins. Shirai *et al.*[26] suggest that this vitamin could play a role in the control of lipid metabolism within rat arterial wall. It must also be noted that Saito *et al.*[27] observed with α-tocopherol and inhibition of the synthesis of cholesteryl esters induced by acetylated LDL in rat peritoneal macrophages.

The administration of liposomal SOD has a marked effect on lipid parameters particularly TCT and TG, whereas the administration of empty liposomes shows no hypolipidaemic effect in control rats. Our results are comparable with those obtained in mice by Olivier *et al.*[28] who tested hypolipidaemic drugs such as nicotinic acid and fibrates. In this similar model, fenofibrate decreased TCT by 40% and TG by 20% at the dose of 100 mg/kg/day for 2 weeks.

In our model PL remained unchanged whatever the diet or treatment used. The lipidogram shows that SOD treatment at both doses normalized VLDL level. The SOD showed a tendency to decrease atherogenic risks by normalizing or increasing HDL. CAR decreased favorably with the higher dose of SOD (1000 μg/kg/day), reaching a value lower than 4, value considered as risk limit of atherogenicity. The increase in HDL by these specific antioxidants is similar to that described by Sandaram *et al.*[29] with rat fed vitamin E as diet supplement.

Likewise, clinical tests of Muckle and Nazir[30] showed a marked effect of α-tocopherol on elevation of the HDL rate for some patients sensitive to this treatment. Thus SOD treatment might decrease the "atherogenic risk" by increasing HDL and decreasing VLDL and CAR. According to our results, liposomal SOD has a clear normolipidaemic action.

Recently Carew *et al.*[31] reported that probucol had an antiatherogenic effect in Watanable hyperlipidaemic rabbits. The effect of probucol is probably due to its antioxidant properties and may be unrelated to the mechanisms by which it lowers plasma cholesterol level. Indeed the structure of probucol is very similar to that of butylated hydroxytoluene, a widely used antioxidant. On the other hand, α-tocopherol administered to HCT subjects has a favourable effect on the lipidogram. It is the main part of natural vitamin E, known for its antioxidant properties.[32] In 1982, Westrope *et al.*[33] had already observed a decrease in TCT and atherosclerosis in Dutch Belted rabbit fed an atherogenic diet but supplemented by 1% of vitamin E. In 1985, an experimental and clinical study with vitamin E diet showed a decrease in plasma lipid peroxides, a favourable effect on the prostacyclin production and platelet aggregability. In patients, this activity was correlated with an antioxidant effect of plasma.[34] Finally, an experiment with the hyperlipidaemic hen developing atherosclerosis showed that a diet supplemented with vitamin E (100 UI/kg) reduced favourably the rate of lipid peroxidation and the atherogenic degree.[23]

These first results are encouraging and should be continued in other studies. It would be interesting to develop a study comparing free SOD and liposomal SOD with

standard hypolipidaemic drugs on other atherosclerosis models. In order to complete this experiment, the serum concentration of lipid peroxides will have to be determined by TBA assay and complemented by a histological examination of hepatic and aortic tissues. In future studies normolipidaemic activity could be estimated by analyzing different fractions of apolipoproteins, using the classification based on the apoprotein profile, proposed by Alaupovic[35] instead of that by Frederickson *et al.*[36] based on lipoprotein concentration levels.

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