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EFFECT OF ATROMID-S ON THE ACTIVITY OF VASCULAR ENZYMES IN RATS

O. MRHOVÁ, D. GRAFNETTER, J. JANDA and J. LINHART

Institute of Clinical and Experimental Medicine, Institute for Cardiovascular Research, Prague, Krč, Budějovická 809, Czechoslovakia

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Abstract—Studies were performed on the effect of Atromid-S on enzymes in the aortic wall and myocardium of rats kept either on standard laboratory diet, or on a high-fat and cholesterol containing diet. In combination with the standard diet Atromid-S seemed to act "favourably" in the aorta, i.e. it enhanced the activities of some glycolytic and Krebs cycle enzymes. In the myocardium the activities of some phosphomonoesterases is significantly reduced.

In combination with the high-fat diet, Atromid-S either exhibited no effect on a majority of the rat aortic enzymes studied, or produced an effect opposite to that seen after consumption of Atromid-S with the standard diet.

In vitro Atromid-S induced an increase in the aortic lactate and malate dehydrogenase activities and a decrease of the 5′-nucleotidase and acid phosphomonoesterase activity.

ATROMID-S (clofibrate, CPIB, ethyl-*p*-chlorophenoxy isobutyrate) is considered to be one of the best hypolipemic agents. Since it has been found useful in prevention and treatment of atherosclerosis and its complications,[1] a number of papers has been published, dealing with clinical and experimental studies of the metabolic effects and the mechanism of action of clofibrate. Particularly detailed studies have been conducted on the effect of Atromid-S on serum lipids, lipoproteins and nonesterified fatty acids, as well as on its influence on lipomobilization.[2–6]

Much less attention has been paid to the effects of Atromid-S on blood and tissue enzymes[7–9] and—to date—practically no attention at all has been paid to the question how Atromid-S influences the metabolism of the vessel wall itself, of the larger tissue in which atherosclerosis develops. The present state of knowledge leaves no doubt that the metabolism of vascular wall is a very sensitive indicator of changes paralleling the development of atherosclerosis, and that an undisturbed energetic metabolism of the vessel walls is one of the primary mechanisms which serve to protect the artery against lipid accumulation and development of atherosclerosis.[10]

Our previous animal experiments, as well as studies in human arteries, have proved that "preatherosclerotic" states are characterized by decrease in the activities of the Krebs cycle and glycolytic enzymes, and on the other hand, an increase in the activity of phosphomonoesterases.[11–14] Consequently, a "favourable" action upon the vascular wall metabolism, such as an activation of the respiratory enzymes, might assumedly increase the vascular resistance against lipid accumulation.

Following this assumption we endeavoured to find out whether Atromid-S affects the enzymatic activities in the arterial wall. In the first phase of this research we

studied the effect of orally administered Atromid-S on the vascular wall enzymes in rats on standard laboratory diet, and a high-fat and cholesterol containing diet, respectively.

MATERIAL AND METHODS

Female Wistar rats, weighing 200–250 g each, were divided into the following groups of 14 animals each : (1) Rats on standard laboratory diet. (2) Rats on standard laboratory diet plus Atromid-S. (3) Rats on high-fat diet (modified Thomas–Hartroft diet:[15] 40% of soya bean oil instead of butter; methylthiouracil omitted; 5% cholesterol content). (4) Rats on high-fat diet plus Atromid-S.

Note. In groups 3 and 4 the high-fat diet had been started 14 days prior to the Atromid-S medication.

Two series of experiments were conducted; in the first Atromid-S was administered for 3 weeks, and in the second, for 6 weeks.

Atromid-S was administered orally once daily using a ball-point injection needle, in doses of 0·1 ml/animal/day. This dosage ensured a constant daily intake and permanent blood levels of Atromid-S.

The rats were killed by decapitation. The aortae were immediately removed, cleaned from periaortic tissue and adventitia, and washed in ice-cold saline. Using a high-speed homogenizer Ultra Turrax (18/2N Janke & Kunkel, GFR), a 1% homogenate from each aorta in 0·9% NaCl solution in normal saline, pH 7, was prepared and kept at 2°. After 1 hr the chilled homogenate was centrifuged at 3000 rev/min for 10 min, and the clear supernatant was immediately tested for the enzyme activities investigated by following methods:

The Alkaline (AP) and Acid (ACP) Phosphomonoesterases were measured using the slightly modified method of Kaplan and Narahara[16] in which disodium phenylphosphate served as substrate and the released phenol is measured by coupling with a diazo reagent. Activity was expressed in μmoles of phenol liberated per hour per 100 μg of protein.

5'-Nucleotidase (5-Nu). Determination of 5'-nucleotidase was performed by a slightly modified procedure of Ahmed and Reis[17] which is mainly based on determining inorganic phosphate released from adenosin 5-monophosphate (AMP). The results were expressed as μg of phosphate liberated per hour per 100 μg of protein in extract.

Adenosintriphosphatase (APP) was estimated by a modification of the method of Banga and Novotny.[18] This method depends on estimation of inorganic phosphate liberated by the enzyme from ATP. The results were expressed as for the previous enzymes.

The group specific carboxylic esterase (Carb. est.) activity was assessed by the slightly modified method of Seligman *et al.*[19] using β-napthol acetate as the substrate. Activity was expressed in μg of β-napthol, liberated per 100 μg of protein extract. Details of the above methods (including the modification) can be found in our previous communications.[10,20]

Malate and lactate dehydrogenases (MDH, LDH) were determined by a technique in which phenazin-methasulphate and neotetrazoliumhydrochlorid served as acceptor of electrons. Activities were expressed in μg of diformazan per 100 μg protein in the aortic extract. For more details see previous work.[12]

Beta-glucuronidase activity was assessed by the method of Talalay *et al.*[21] with the slight modifications of Dyrbye and Kirk.[22] Enzyme activities were expressed in μg of phenolphthalein liberated per 100 μg protein in the extract per 16 hr.

Protein content in aortic extracts was determined by the method of Lowry.[23]

The above described methods were also used during the *in vitro* experiments. However, the incubation media were enriched for the necessary concentrations of Atromid-S by dissolving the sodium salt of ethyl-*p*-chlor-phenoxyisobutyrate.

RESULTS

1. In the aorta of rats kept on the standard laboratory diet, Atromid-S elicited a statistically significant increase in the activity of carboxyl esterase. After 3 weeks of medication, this increase could not be observed (Figs. 1 and 2). The LDH and MDH

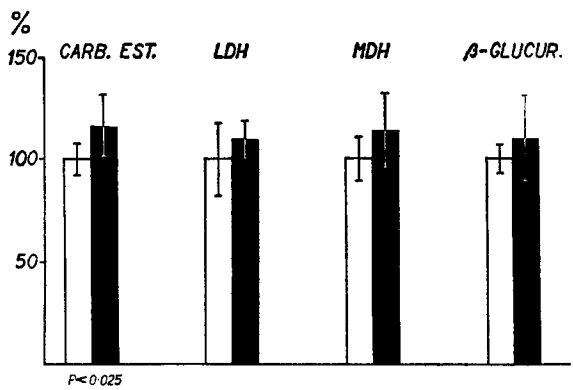

FIG. 1. The effect of Atromid-S on aortic carb. esterase, lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and β-glucuronidase activities in rats kept on standard laboratory diet. Empty columns, control rats. Black columns, Atromid-S treated rats (400 mg/kg/day for 3 weeks). Results are expressed as percentage differences, taking the mean activity of controls as 100 per cent.

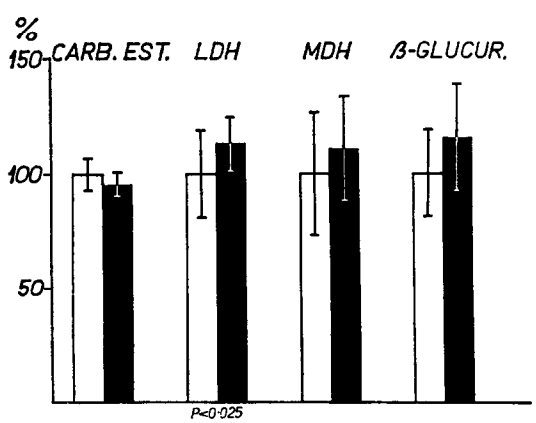

FIG. 2. The effect of Atromid-S on aortic carb. esterase, lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and β-glucuronidase activities in rats kept on standard laboratory diet. Empty columns, control rats. Black columns, Atromid-S treated rats (400 mg/kg/day for 6 weeks). Results are expressed as percentage differences, taking the mean activity of controls as 100 per cent.

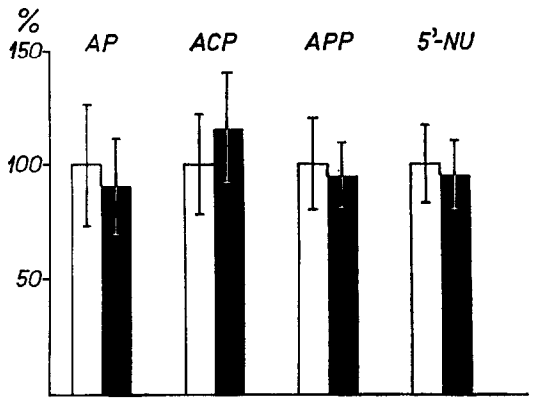

FIG. 3. The effect of Atromid-S on aortic Alkaline phosphatase (AP), Acid phosphatase (ACP), adenylpyrophosphatase (APP) and 5-nucleotidase activities in the same animals as in Fig. 2.

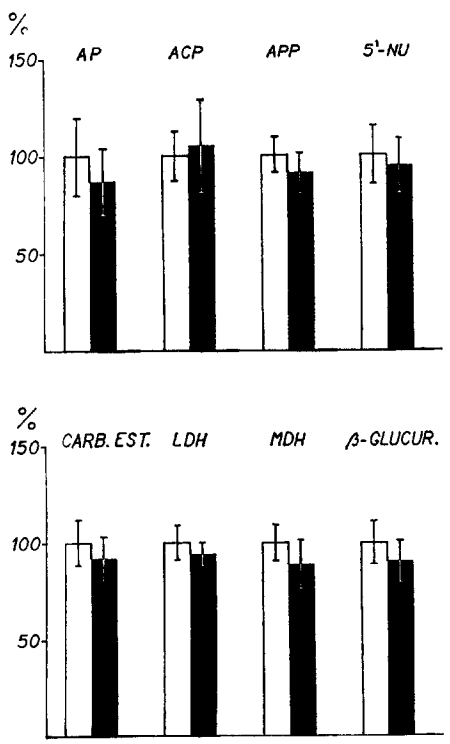

FIG. 4. The effect of Atromid-S on aortic carb. esterase, lactate dehydrogenase (LDH), malate dehydrogenase (MDH), β-glucuronidase, alkaline phosphatase (AP), acid phosphatase, (ACP) and 5'-nucleotidase activities in rats kept on a high-fat cholesterol containing diet. Empty columns, control rats. Black columns, Atromid-S treated rats (400 mg/kg/day for 3 weeks). Results are expressed as percentage differences, taking the mean activity of control as 100 per cent.

activities tended to increase after 3 weeks of Atromid-S administration. After 6 weeks this increase reached statistical significance in the case of LDH, whereas in the case of MDH it remained nonsignificant because of a great variability (Figs. 1 and 2).

The ACP and β-glucuronidase exhibited a moderate tendency to increased activities, mainly after 6 weeks of Atromid-S medication (Figs. 2 and 3).

The activities of 5-Nu, AP, and APP remained almost uninfluenced in both experimental series.

2. Atromid-S administered to rats on the high-fat diet exerted an influence on the activities of most rat aortic enzymes studied, in contrast to what was seen in rats fed the standard laboratory diet.

After 3 weeks of Atromid-S medication, a nonsignificant decreasing tendency was found in the activities of carboxyl esterase, LDH, MDH, AP, APP, β-glucuronidase, and 5-Nu (Fig. 4).

On the other hand, after 6 weeks the LDH activity also showed an increasing tendency; consequently even in combination with the high-fat load, Atromid-S stimulated the LDH activity (Fig. 5).

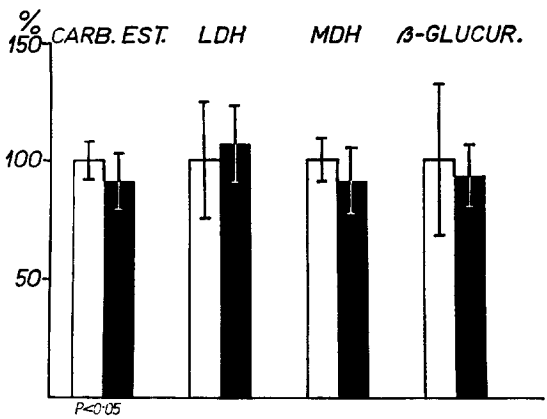

FIG. 5. The effect of Atromid-S on aortic carb. esterase, lactate dehydrogenase (LDH) malate dehydrogenase (MDH) and β-glucuronidase in rats kept on a high-fat cholesterol containing diet. Empty columns, control rats. Black columns, Atromid-S treated rats (400 mg/kg/day for 6 weeks) Results are expressed as percentage differences, taking the mean activity of controls as 100 per cent.

3. Tests performed with the Atromid-S *in vitro* (at concentrations of 10, 100 and 1000 μg of sodium salt of ethyl-*p*-chlor-phenoxyisobutyrate per milliliter) revealed an activation of LDH and MDH, a marked decrease in the activities of acid phosphatase and 5-Nu, and a moderate inhibition of alkaline phosphatase in the homogenates prepared from normal aortae. The activities of APP and carboxyl esterase remained uninfluenced by Atromid-S *in vitro* (Table 1).

4. After 6 weeks of Atromid-S medication the myocardium of rats on standard laboratory diet showed significantly decreased activities of alkaline phosphatase and

TABLE 1. *In vitro* EFFECT OF ATROMID-S ON ENZYME ACTIVITIES CONTAINED IN 1% AORTIC EXTRACT IN RATS

Additions per ml of medium	Mean extinction value (relative enzyme activity)						
	AP	ACP	5-Nu	APP	Carb. ester.	LDH	MDH
None	0·910	0·119	0·299	0·330	0·320	0·451	0·290
10 μg ATR.	0·925	0·58	—	0·330	—	0·420	0·320
100 μg ATR.	0·915	0·57	0·172	0·329	0·299	0·460	0·340
1000 μg ATR.	0·933	0·87	0·155	0·331	0·299	0·590	0·350

ATP-ase. The activities of the other enzymes investigated in the rat myocardium remained substantially uninfluenced by Atromid-S (Fig. 6).

DISCUSSION

Certain pathologic states and situation factors connected with the development of atherosclerotic changes (e.g. calciferol intoxication, alloxan-induced diabetes, accumulation of mucopolysacharides, hypothyroidism, cholesterol feeding, etc.) reduce the vascular esterolytic activity, thus facilitating lipid accumulation in the vascular wall as well as abnormal transport conditions of blood lipids. Therefore, an elevation of the esterolytic activity by Atromid-S would represent, in a certain respect, a fortification of one of the vascular protective systems against the lipid accumulation. Since, however, after 6 weeks of Atromid-S administration this increased esterase activity disappeared, the question remains open whether the increase in the activity, recorded after 3 weeks of medication, was not just a transient compensatory reaction.

In rats kept on the standard laboratory diet, the activities of aortic LDH and MDH were influenced by Atromid-S in a "favourable" sense, i.e. exhibited a tendency to increase, although the high variability did not allow a proof of statistical significance after 3 weeks. However, after 6 weeks the group of standard diet plus Atromid-S exhibited a significant increase in the LDH activity.

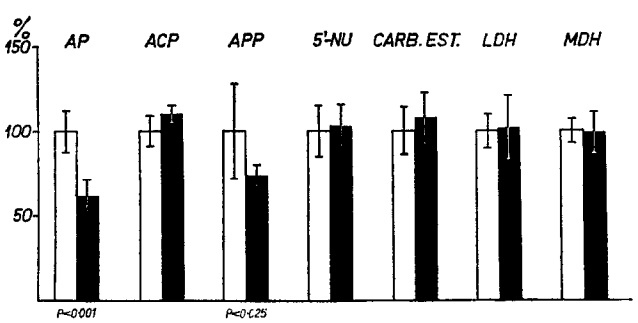

FIG. 6. The effect of Atromid-S on heart enzyme activities of rats kept on standard laboratory diet. Empty columns control rats. Black columns, Atromid-S treated rats (400 mg/kg/day for 6 weeks. Results are expressed as percentage differences, taking the mean activity of controls as 100 per cent.

In vitro experiments proved that Atromid-S, particularly at the higher concentrations, elevated the activity of LDH and MDH.

Therefore, Atromid-S might be inferred to influence "favourably" the enzymes mentioned above. This might be of considerable importance in the light of the fact that a well functioning respiratory enzyme series represents an important protective factor of the vessel wall.

Although *in vitro* experiments revealed an inhibition of the AP, 5-Nu, and particularly ACP activities by Atromid-S at all concentrations tested, the *in vivo* activities of these enzymes in the aortae of rats kept on the standard diet showed no major change.

Our previous studies[13] revealed a certain connection between the changes in the activity of phosphomonoesterases and those in the reaction of the vascular connective tissue; nevertheless, Atromid-S which had been observed to influence blood lipid concentrations and also lipids in certain tissues, does not influence the aortic phosphomonoesterase *in vivo*. On the other hand, however, in the myocardium of the experimental animals the AP and ATP-ase activities were very markedly reduced.

Atromid-S, administered to rats kept on the high-fat diet (which later alone elicits no essential changes in the activities of the enzymes studied), produced in a majority of the studied enzymes a trend opposite to that one seen in rats which were fed normal laboratory diet. These results are in good agreement with the work of Grafnetter *et al.*,[25] who in parallel experiments found a significant increase in the cholesterol and phospholipid contents of the aorta of rats kept on the high-fat diet supplemented with Atromid-S.

Thus the tendency to a decrease in the activities of LDH and MDH in this group may be a manifestation of the increase in the aortic lipid levels.[13]

Nevertheless, after 6 weeks of Atromid-S administration, an increasing tendency of the LDH activity was apparent.

On the basis of our results, Atromid-S may be inferred to exert some influence on the proper metabolism of the vascular wall.

However, the diet abnormally high in the fat and cholesterol seems to affect the influence of Atromid-S on the vessel wall metabolism in an unfavourable way.

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