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THE ANTICOAGULANT, HEPATIC LIPASE-RELEASING AND LIPOPROTEIN LIPASE-RELEASING ACTIVITIES OF SEVERAL NATURAL AND CHEMICALLY MODIFIED HEPARINS DIFFER

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Abstract—Several 'natural' heparins have been found to have different potencies for releasing hepatic lipase and lipoprotein lipase. These differences can also be obtained by treating heparins with physical and chemical methods, which also affect the anticoagulant activity. These differences in potency in hepatic lipase-releasing activity are discussed in terms of the role of this lipase in lipoprotein and cholesterol metabolism.

Until now interest has been concentrated on the overall ability of a heparin to release total lipase activity into the blood stream after i.v. injection [1–3], with no distinction being made between lipoprotein lipase or hepatic lipase-releasing potencies. At the present time, there is increasing evidence that hepatic lipase has an important role in metabolism of lipoproteins [4–7]. Jansen and Hülsmann [8] have proposed that hepatic lipase is involved in the delivery of cholesterol to the liver or other lipase-containing tissues. In their model, cholesterol is taken up from peripheral cells by a phospholipid-rich HDL and subsequently delivered to the liver via a mechanism involving the depletion of phospholipid from HDL by hepatic lipase. Recently it has been shown that the uptake of high-density lipoprotein cholesterol by hepatoma cells is directly related to the extent of HDL phosphatidylcholine hydrolysis, which in turn depends on the phospholipase activity of hepatic lipase [7].

The aim of this study was to see whether different heparins have different potencies in releasing hepatic lipase and lipoprotein lipase into the blood stream and if these activities can be manipulated since:

(1) Heparins are heterogenous (for a recent review see ref. [9]) and can be separated into at least two fractions by electrophoresis in barium acetate buffer on either cellulose acetate [10] or on agarose gel [11]. These two fractions are called slow-moving (SM) heparin and fast-moving (FM) heparin because of the noteworthy differences in their electrophoretic mobilities. The heterogeneity of heparins has also been shown by fractional precipitation of their barium salts [12]. This last method has been used in this study to prepare substantial amounts of heparins which were characterized according to their electrophoretic mobilities in barium acetate buffer as SM or FM heparin (see Materials and Methods).

(2) Heparins can undergo periodic oxidation. The products resulting from this process retain either no or very little anticoagulant activity, while the lipase-releasing activities are not lost [13, 14]. We have applied this process to SM and FM heparin.

MATERIALS AND METHODS

Heparins. The heparins used were: Hep-1, a pig mucosal heparin prepared by Professor Vercellotti in V-labs, Covington, U.S.A. from a mixture of GAGs supplied by Crinos; Hep-2, a pig mucosal heparin supplied by Terhormon, Novara, Italy, batch 78/78; Hep-3, a pig mucosal heparin supplied by Terhormon, batch 185/80; Hep-4, a low molecular weight heparin supplied by Opocrin, Carlo, Italy, batch 23; Hep-5, a pig mucosal heparin from Diosynth, Oss, The Netherlands, batch Hb 1659-I, the proposed European Pharmacopoeia standard. This sample was kindly supplied by Dr E. A. Johnson of the National Institute for Biological Standards and Control, London, U.K.; SM-Hep-3 and FM-Hep-3, these are the SM and FM fractions of heparin Hep 3. These fractions were prepared in the Chemical Research Laboratories of Crinos, as described under Preparation of SM and FM heparins; RO-SM-3 and RO-FM-3, these are the oxidized SM and FM heparins which were stabilized by reduction. They were prepared in the Chemical Research Laboratories of Crinos, as described under Preparation of oxi-reduced heparins.

Preparation of SM and FM heparins. Heparin sodium salts were transformed into barium salts by treatment with Amberlite IR 120 (H^+ form), in water at 4°, followed immediately by neutralization with 2 M barium hydroxide. The solution–suspension obtained was concentrated to a final 5% (w/v) heparin concentration and left to stand overnight at 4°. The insoluble material was removed by centrifugation (1500 *g*) and ethanol added stepwise to the supernatant to obtain the following ethanol concentrations (v/v): 8%, 15.4% or 20%. Each ethanol solution was left to stand overnight at 4° and the

unsoluble material separated by centrifugation, before the next ethanol addition. Aliquots of four different fractions (the last was the material soluble in 20% ethanolic) were electrophoresed (after transformation of small amounts into the sodium salts) and the material precipitated from the 8% ethanol solution was seen to be SM heparin and that soluble in 20% ethanol was FM heparin. The intermediate material was discarded. Each fraction was converted into its sodium salt by treatment with Amberlite IR 120 (Na^+ form) and then with Duolite ESS 466 (Na^+ form). The yields were 37.1% for SM and 26.1% for FM heparins.

Preparation of oxi-reduced heparins. Heparins were oxidized by sodium periodate in an aqueous medium at 4° for 24 hr. The reaction was stopped by addition of ethylene glycol. The oxidized heparin was dialyzed with several changes of distilled water. The dialyzed oxi-heparin solution was reduced by gradual addition of sodium borohydride checking continuously both pH and temperature. The final solution was concentrated at reduced pressure and lyophilized. The yield was about 90% [13].

Analytical methods. Sulfur content was determined gravimetrically as barium sulfate. The hexosamines were determined by the Elson–Morgan colorimetric method as described by Boas [15], using glucosamine as the standard. The uronic acids were determined by the colorimetric carbazole–borate method [16], with glucuronic acid as reference. The ratios of carboxyl-to-sulfate groups were determined by conductimetry [17]. Optical rotations were measured in a Perkin–Elmer Model 141 polarimeter. Electrophoresis was done on agarose gel in barium acetate buffer [11]. Gel filtration chromatography was used for the determination of mean mol. wts of the heparins under investigation, as in ref. [18], using heparin fractions of known mol. wts kindly supplied by Dr E. A. Johnson.

Animals. Male rats (Charles River, Calco, Italy) weighing 200 ± 10 g were used throughout.

Determination of lipase activities. Heparins were injected i.v. into rats and the animals killed 10 min later. The anticoagulated blood was centrifuged at 4° to obtain plasma, and plasma total lipase activity (lipoprotein lipase plus hepatic lipase) was determined as previously described [19], using Intralipid as the substrate [20]. Hepatic lipase was evaluated with Intralipid in the same plasma samples in the presence of 1 M NaCl, as previously reported [19], or with phosphatidylcholine as the substrate, in view of the findings of Bamberger *et al.* [7] that the uptake of HDL cholesterol by hepatoma cells was directly related to the extent of HDL phosphatidylcholine hydrolysis by hepatic lipase and also taking into account the method of Vogel *et al.* [21] and the observations of Zieve [22]. Lipoprotein lipase activity was calculated by subtracting hepatic lipase activity (determined with Intralipid) from total lipase activity.

Determination of anticoagulant activities. Anticoagulant activities of heparins were assayed *in vitro* (sheep plasma) as described in the United States Pharmacopoeia XX, National Formulary XIV and expressed as 'International Units' (IU/mg). The evaluation of anti Xa activities (U/mg) was performed *in vitro* (sheep plasma) using Sigma Kit no. 870-B which is essentially based on the method of Yin *et al.* [23]. In all assays the standard heparin was Hep-3, the anticoagulant activity of which was previously determined against the Third International Standard Heparin supplied by the National Institute for Biological Standards and Control, London, U.K.

Statistical analyses. Hep-3 was arbitrarily used as the standard for determination of relative lipolytic potencies and was assigned a potency of one for each lipase and each substrate type. Relative potencies for each kind of lipase and for each kind of substrate were calculated as described by Tallarida [24], using an HP 86 B computer.

RESULTS

Table 1 lists the physicochemical parameters of the 'natural' and chemically modified heparins, that is the mean mol. wts, the content of sulfur, hexosamine and uronic acid, the ratio of sulfate to carboxylate, the ratio of uronic acids and the optical rotations. Hep-4 had the lowest content of sulfur and

Table 1. Physicochemical parameters of 'natural' and chemically modified heparins

Heparin	Mean mol. wt	Sulfur (%)	Hexosamine (%)	Uronic acid (%)	Ratio sulfate to carboxylate	Ratio of uronic acids*	$[\alpha]_D^{20}$
Hep-3	12,000	11.19	25.4	38.7	1.83		+52.7
Hep-1	17,800	10.92	21.8	29.2	2.17		+43.6
SM-Hep-3	18,600	11.48	23.7	39.9	2.05	3.93	+53.8
FM-Hep-3	11,500	11.00	23.8	36.8	1.80	1.79	+48.0
RO-SM-3	15,200	11.74	24.0	31.8	2.02		+63.5
RO-FM-3	11,500	10.94	23.9	23.6	1.84		+64.2
Hep-2	14,000	10.23	N.D.	N.D.	1.90		+49.0
Hep-5	12,000	12.31	N.D.	N.D.	2.15		+52.5
Hep-4	4,500	9.55	19.7	26.3	1.94		+42.9

N.D., not determined.

* This is the ratio of the amount of uronic acids resistant to periodate oxidation to the amount of uronic acids which undergo the periodate oxidation (evaluated by the difference between total initial uronic acids and total final uronic acids).

Table 2. Relative potencies of the 'natural' and chemically modified heparins. Hep-3 was arbitrarily taken as the standard and assigned a potency of one for each type of lipase and each type of substrate. The anticoagulant activities of the heparins were assayed with the Third International Standard Heparin as reference standard

Heparin	Total lipase relative potency (Intralipid)	Lipoprotein lipase relative potency (Intralipid)	Hepatic lipase relative potency (Intralipid + 1 M NaCl)	Hepatic lipase relative potency (Phosphatidylcholine)	Anticoagulant activity (IU/mg)	Anti Xa activity (U/mg)
Hep-3	1	1	1	1	151.53	151.53
Hep-1	1.59	1.65	1.17	1.37	212.97	148.80
SM-Hep-3	1.32	1.38	1.04	0.93	144.13	163.50
FM-Hep-3	0.67	0.65	0.50	0.67	129.98	82.00
RO-SM-3	1.51	1.54	1.22	1.42	43.37	50.54
RO-FM-3	0.85	0.87	0.83	0.88	10.54	22.30
Hep-2	0.69	0.77	0.40	0.69	161.80	167.00
Hep-5	1.14	1.18	1.00	1.02	188.50	169.00
Hep-4	0.52	0.58	0.35	0.51	48.40	63.40
Ratio between the highest and the lowest potency	3.06	2.84	3.47	2.78	20.20	7.60

hexosamine and the lowest mol. wt. SM-Hep-3 had the highest mol. wt. When SM-Hep-3 and FM-Hep-3 were oxi-reduced the content of uronic acid decreased while the optical rotation increased.

Table 2 summarizes the relative potencies of the same heparins reported in Table 1.

From the data reported in Table 2 it can be seen that different heparins have different relative potencies for inducing the release of the different lipases into the blood stream. The range of the variation was from 2.78 to 3.47 times (Table 2). For the relative potencies in inducing lipases, the most outstanding heparins were: Hep-1, a 'natural' heparin (Table 2), and RO-SM-3, which perhaps we could call a chemically modified heparin (Table 2), while Hep-4 had the worst activities (Table 2). SM-Hep-3 was more potent than Hep-3 in relative potency of total lipase and lipoprotein lipase (Table 2), but was practically equipotent with Hep-3 for the two hepatic lipase activities (Table 2). When RO-SM-3 was generated from SM, its relative potencies were still greater, in comparison with both Hep-3 and SM-Hep-3. FM-Hep-3 was less potent than Hep-3 or SM-Hep-3 (Table 2). When FM heparin was modified chemically into RO-FM-3, this latter had higher relative potencies (Table 2).

The correlation between the relative potencies of hepatic lipase determined with Intralipid in the presence of 1 M NaCl and the relative potencies of hepatic lipase release determined with phosphatidylcholine, using data from Table 2, was highly significant ($P < 0.001$). The correlation between relative potencies of total lipase and the sulfate/carboxylate ratio was significant ($P = 0.027$), as was the correlation between relative lipoprotein lipase potencies and the sulfate/carboxylate ratio ($P = 0.018$). The correlations between both hepatic lipase relative potencies and the sulfate/carboxylate ratio were not significant. The correlations between the mol. wts and the relative potencies of total lipase, or of lipoprotein lipase, or of hepatic lipase, determined with Intralipid in presence of 1 M NaCl, or of hepatic lipase determined with phosphatidylcholine were $P = 0.011, 0.010, 0.048, 0.049$, respectively.

The outstanding heparins for anticoagulant activity were Hep-1 and Hep-5 (Table 2). Hep-4 had the worst activity (Table 2). When SM-Hep-3 and FM-Hep-3 were oxidized and reduced their anticoagulant activities were reduced, but the activity of FM-Hep-3 was perhaps affected more than that of SM-Hep-3 (Table 2). When the two RO-Heparins were excluded from the calculation a significant ($P = 0.041$) correlation between the mol. wts and the anticoagulant activities (USP) was obtained. Operating in the same way for the correlation between the mol. wts and the anticoagulant activities (anti Xa) an improvement in the correlation was obtained, but it was not significant.

DISCUSSION

As far as the 'natural heparins' are concerned, all data (i.e. mol. wts, the content of sulfur, hexosamine and uronic acid, the ratio of sulfate to carboxylate, the ratio of uronic acids and the optical rotations) closely approached those found for purified com-

mercial heparins, except the Hep-4 content of sulfur and hexosamine (Table 1). The mol. wt of this last heparin was very low (Table 1).

The behavior of Hep-3, giving rise to a slow-moving and a fast-moving component, when electrophoresed in barium acetate buffer, was similar to those of purified commercial heparins. When SM-Hep-3 and FM-Hep-3 (prepared by ethanol precipitation of barium salts) were compared to parent Hep-3 by electrophoresis in barium acetate, they matched the 'slow-moving' and 'fast-moving' components, respectively. The mean of the mean mol. wts of SM-Hep-3 and FM-Hep-3 did not approach the mol. wt of parent Hep-3, while the mean of the mean mol. wts of RO-SM-3 and RO-FM-3 approached more closely that of parent Hep-3 (Table 1). This depended on the high mol. wt of SM-Hep-3 in comparison to RO-SM-3, while the mol. wts of FM-Hep-3 and RO-FM-3 matched each other (Table 1). As a consequence, we can perhaps state that the oxidation by periodate did not affect the mol. wt of heparins SM-Hep-3 and FM-Hep-3. The high value of SM-Hep-3 mol. wt could be explained by hypothesizing that this heparin, isolated as insoluble barium salt, could behave in a different way when processed by gel-chromatography.

Actually the separation of slow and fast components of a heparin by electrophoresis in barium acetate buffer cannot be explained taking into account just the mol. wt because the differences between the electrophoretic mobilities are greater than those between the mol. wt. Recently it was shown that the electrophoretic mobilities of heparins are affected by electric charge densities [9]; more exactly the electrophoretic mobilities of biopolymers depend on the ratio of the number of electrical charges to mass (Z/m) or mole (Z/M).

SM-Hep-3 showed a higher content of sulfate groups as well as of uronic acids (carboxylate groups) in comparison to FM-Hep-3 (Table 1). The values of Z/m and Z/M of the former heparin were 0.55 and 101.4, respectively, while those of the latter were 0.52 and 59, respectively. Therefore, FM-Hep-3 should have had a slower electrophoretic mobility than that of SM-Hep-3, while in barium acetate buffer paradoxically the opposite happened. This finding could be explained considering that the barium ion causes the precipitation *in situ* of the 'slow-moving' component which is no more affected by the electrical field. So it happens that an artifact, caused by an interaction between the barium acetate buffer and the 'slow-moving' component is explained as a mere difference in mol. wt, when a commercial heparin is fractionated into its slow and fast components. Anyway the most outstanding chemical–physical variations, caused by the oxi-reduction procedure, concern the content of uronic acid and the optical rotation. The former is a predictable consequence of the chemical treatment, the latter could be explained as the result of the opening of pyranose rings by periodate, which could affect the interaction of heparin chain with the polarized light. The ratios of the uronic acids of SM-Hep-3 and FM-Hep-3 were different (Table 1) indicating that SM-Hep-3 and FM-Hep-3 were unlike heparins. This ratio could be an interesting and useful index to estimate the ratio of L-iduronic-2-sulfate acid (the only uronic acid which does not undergo periodate oxidation [14]) to non-sulfate uronic acid into the heparin chain.

The different heparins had different potencies for releasing into the blood stream not only total lipase activity but also lipoprotein lipase and hepatic lipase. These potencies differed by a factor of 2.78–3.47 (Table 2). Some 'natural' heparins such as Hep-1, have of themselves high relative potencies for releasing lipases into blood stream. Using separation techniques and chemical modifications, it was possible to obtain a heparin (RO-SM-3) whose activities in releasing lipases were very close to those of Hep-1 (Table 2). But this last chemically modified heparin (RO-SM-3) did not have the very high anticoagulant activity of Hep-1. This demonstrated that it is possible to separate to some degree in RO-SM-3 the two activities [13] although the active site for antithrombin [2, 25, 26] contains at least one disaccharide unit which can bind also to lipases [2]. The same was also true for RO-FM-3 (Table 2). FM-Hep-3 had an anticoagulant activity lower than that of SM-Hep-3 (−10%, Table 2) but its potencies in inducing lipases were still lower than those of SM-Hep-3 [lipoprotein lipase (−53%, Table 2), hepatic lipase determined with Intralipid + 1 M NaCl (−52%, Table 2) and hepatic lipase determined with phosphatidylcholine (−28% Table 2)]. When FM was transformed into RO-FM-3, its lipase-releasing potencies increased [lipoprotein lipase (−37%, with a 16% gain, Table 2), hepatic lipase with Intralipid + 1 M NaCl (−20%, with a 32% gain, Table 2), and hepatic lipase with phosphatidylcholine (−6%, with a 23% gain, Table 2)]. At the same time RO-FM-3 anticoagulant activity was decreased more than that of RO-SM-3 (Table 2). We can therefore hypothesize that the inactivation of the sites for antithrombin of FM-Hep-3 (−10% of those in SM-Hep-3) made the trisulfate disaccharide sequences [2] more available for binding to lipases. The opinion that splitting the C_2–C_3 bonds of non-sulfated uronic units does not affect lipase release potency [13] of a heparin is thus not strictly correct.

The very high correlation between results with the two methods for determination of hepatic lipase suggests that we measured two enzymatic activities of the same enzymatic protein, i.e. hepatic lipase. The correlations between total lipase (mainly lipoprotein lipase) or lipoprotein lipase relative potencies and the ratio of sulfate to carboxylate could be explained on the basis that this ratio essentially parallels the 'concentration' of the disulfated trisaccharide units which preferentially bind to the lipases [2]. The low activity of Hep-4, could be explained taking into account its low mol. wt. In fact there are studies demonstrating the reduced ability of heparin to bind to and release lipase enzymes as the mol. wt of heparin decreases [27, 28]. At the moment we have no explanation for the lack of correlation between hepatic lipase activities and the sulfate/carboxylate ratio. Perhaps the binding between hepatic lipase and heparin takes place at different 'sites' on heparin from those of the binding of lipoprotein lipase.

The elimination of the active site for antithrombin

in RO-SM-3 and RO-FM-3 could be explained because a significant correlation, between the mol. wts and the anticoagulant activities (USP), was obtained when these two heparins were excluded from the calculation. The lack of correlation between the molecular weights and the anticoagulant activities (anti Xa) may perhaps be due to different requirements for formation of ternary complexes with different proteinases [9], requirements not affected by the chain length of the polysaccharide. The low activity of Hep-4 could be explained by taking into account that a heparin, which is a poor releaser of lipase, is also poor in its anticoagulant activity [35] and that low mol. wt heparins with low anti Xa activities have been described [36].

In view of the growing body of evidence for an important role for hepatic lipase in metabolism of lipoproteins and cholesterol catabolism [4–8], and since in survivors of myocardial infarction intermediate density lipoproteins (IDL) are higher than in controls [29], moderately increased levels of IDL cholesterol are closely associated with a high frequency of coronary artery disease [30] and IDL has been proposed as a cause of atherogenesis [31]. As IDL are specific substrates for hepatic lipase [32–34], and inhibition of hepatic lipase leads to accumulation of IDL in plasma [34], we wonder if chemically modified heparins and/or those with concentrated particular structural 'features', might be used to affect specifically hepatic lipase and hence modify metabolism of IDL, high-density lipoproteins and cholesterol.

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