Relationship of Lipolytic and Esterolytic Activity of the Aorta to Susceptibility to Experimental Atherosclerosis

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Aortic lipolytic (lipoproteinolytic) and esterolytic activity of various species were investigated by biochemical and histochemical methods. Lipolytic and esterolytic activities in the aorta of species susceptible to experimental atherosclerosis are lower than in the rat, a very resistant species. The histochemical technics permitted the localization of enzymatic activity in individual layers of vessel wall.

THE VARIED susceptibility of different species to spontaneous and experimental atherosclerosis is one of the significant problems in the study of this disease. Previous work has shown that rat aorta has a high lipolytic (lipoproteinolytic) activity (estimated in terms of the release of unesterified fatty acids from human lipemic serum) as opposed to that of other species more susceptible to atherosclerosis. As is well known, the rat is very resistant to the ordinary technics of producing experimental atherosclerosis.

The introduction of a histochemical method for the qualitative and quantitative estimation of nonspecific esterase in tissues has enabled a comparative study of species differences in lipolytic and esterolytic activity of aorta. This method also enables localization of enzymatic activity in individual layers of the vascular wall.

Histochemical estimation of nonspecific (simple)* esterase in human and mammalian aortas has been carried out previously by Gomori’s method, and by an indoxyl method. There is some disagreement between the cited findings, some authors finding no activity at all in the intima and media, others reporting a greater or lesser degree of activity. Qualitative and quantitative species differences in aortas have not been reported by these authors.

METHODS

Biochemical Estimation of Lipolysis. Thirty-one rats of the Wistar strain, 16 rabbits, 6 golden hamsters, 6 guinea pigs, and 4 Leghorn cocks were used, all males between four and seven months of age, to rule out the effects of aging. The method of estimation of lipolytic activity consisted basically in incubating lipemic human serum (diluted 1:1 with Sorensen phosphate buffer at pH 7.38) with a carefully weighed and finely divided sample of freshly prepared aorta. In comparative experiments, the amount of tissue used was kept fairly constant as far as possible. Incubation was at 37°C for 150 minutes, and the degree of lipolysis was estimated by means of freed unesterified fatty acids measured in the incubating medium by Dole's method. One milliliter samples were taken of the incubating medium at 7 and 150 minutes, added to 5 ml. of extraction mixture (isopropyl alcohol, heptane and H2SO4 in a ratio of 40:10:1) in a measuring cylinder with a ground glass stopper, and the contents were well shaken. After about 10 minutes, 3 ml. heptane and 2 ml. distilled water were added, and the mixture was once again shaken. After the 2 phases separated, 3 ml. were pipetted off from the upper layer into a test tube for titration. Titration was carried out in the presence of 1 ml. indicator solution (Xine blue in 90 per cent alcohol) with a microburette containing 0.018N NaOH. The results were calculated as mEq./L./Gm. tissue (table 1). For comparison of results from different experimental days, the data were expressed percentagewise, rat tissue.
TABLE 1.—Lipolytic Activity of Rat, Rabbit and Guinea Pig Aorta, Using the Same Hyperlipemic Serum as Substrate

<table>
<thead>
<tr>
<th>Species</th>
<th>Aorta wgt. (mg.)</th>
<th>NEFA (mEq.)</th>
<th>NEFA (mEq./L.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>63.5</td>
<td>0.12</td>
<td>1.90</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>61.0</td>
<td>0.05</td>
<td>0.81</td>
</tr>
<tr>
<td>Rabbit 3</td>
<td>62.0</td>
<td>0.07</td>
<td>1.12</td>
</tr>
<tr>
<td>Guinea pig 1</td>
<td>47.0</td>
<td>0.24</td>
<td>5.10</td>
</tr>
<tr>
<td>Guinea pig 2</td>
<td>58.0</td>
<td>0.21</td>
<td>3.62</td>
</tr>
<tr>
<td>Rat 1</td>
<td>60.0</td>
<td>0.59</td>
<td>9.83</td>
</tr>
<tr>
<td>Rat 2</td>
<td>61.0</td>
<td>0.61</td>
<td>10.00</td>
</tr>
<tr>
<td>Rat 3</td>
<td>60.0</td>
<td>0.99</td>
<td>16.50</td>
</tr>
<tr>
<td>Rat 4</td>
<td>59.0</td>
<td>0.68</td>
<td>11.52</td>
</tr>
<tr>
<td>Rat 5</td>
<td>35.0</td>
<td>0.28</td>
<td>8.00</td>
</tr>
</tbody>
</table>

*Experimental conditions are the same as in figure 1. NEFA represent the differences in the amount of freed unesterified fatty acids between the 7 min. and 150 min. incubation samples, expressed either in terms of the total sample or per gram of sample.

values being set arbitrarily at 100 per cent (fig. 1). Since the hyperlipemic substrate was not constant on each experimental day, aortas from a representative group of all the species used were incubated in each single experiment along with rat aortas.

Histochtmical Estimation of Nonspecific Esterase. The aortas of 7 rabbits, 5 guinea pigs, 4 golden hamsters, and 16 rats were used. The tissue was removed after killing the animals, and fixed both in cold formalin-calcium chloride solution (Baker's solution) for four hours and in cold acetone for 24 hours. Tissues fixed in Baker's solution were sectioned on a freezing microtome at 15 μ. Tissues fixed in acetone were embedded in paraffin in the standard manner and sectioned at 10 μ. Ten sections were taken from each part of the aorta. Nonspecific esterase and AS-esterase were detected by an azo-coupling method. The sections were incubated in a buffer solution of alpha-naphthol acetate or naphthol-AS acetate in the presence of the tetrazonium-fluoroborate of o-dianisidine. An azo dye developing enzymatic activity developed, black with alpha-naphthol, blue with naphthol AS.

A quantitative estimation of activity was also carried out with rat and rabbit tissue. This consisted of a photometric estimation of the azo dye resulting from the coupling of alpha-naphthol with the diazonium-fluoroborate of 5-chloro-2-toluindine. The azo dye is extracted from the sections with dioxane.

RESULTS

Lipolytic Activity of the Aorta, Estimated Biochemically

Table 1 shows a representative example of lipolytic activity of rat, rabbit, and guinea pig aorta, estimated on the same day with the same lipemic substrate (from the same hyperlipemic donor). Figure 1 summarizes the results of all comparative experiments in which the tissue activity of each species was compared to rat tissue activity. Each individual value is from a different animal. The lipolytic activity of rat aorta is significantly greater than that of rabbit aorta (p < 0.001).

There was also a marked difference in activity of cock (p < 0.01) and guinea pig aorta (p < 0.001) in comparison with the rat. In the hamster there was a tendency to lower activity values than in the rat, but the differences were not significant in this small group.

Histochtmical Estimation of Esterase

The localization of esterases and AS-esterases was always the same along the course of
FIG. 2. Nonspecific esterase in rat aorta. There is a very intense reaction in the media, but a weak reaction in the intima. Elastic fibers are unstained. Adventitia cannot be seen in this preparation. In this and subsequent figures, the sections were frozen, 15 μ thick, cut from tissues fixed in cold formaldehyde-chloride (4 hrs.). Substrate was alpha-naphtol acetate, with tetrazonium fluoroborate of o-dianisidine as the coupling agent. Incubation time 20 min. Magnification 170X. i = intima, m = media, a = adventitia.

FIG. 3. Nonspecific esterase in rabbit aorta. The reaction is more intense in the outer part of the media, in elements lying between the unstained elastic membranes. There is practically no staining of the intima. The adventitia cannot be seen here.

FIG. 4. Nonspecific esterase in guinea pig aorta. There is a positive reaction in the endothelial cells, particularly in the bottom of endothelial folds and intimal fibrocytes. Reaction in media weak, but branched adventitial fibrocytes reacted strongly.

FIG. 5. Nonspecific esterase in golden hamster aorta. Reaction in intima weak, reaction in adventitial fibrocytes intense. No reaction in media.

the aorta in a given species. There were, however, marked interspecies differences.

**Qualitative Differences.** In rats (fig. 2) there was a very intense reaction after a five minute incubation of frozen sections, and a 15 minute incubation of paraffin sections, particularly in the media in the layers between the elastic membranes. The intima reacted only occasionally. In the adventitia there were branched connective tissue cells with a marked positive reaction. In intercostal arteries there was a reaction only in the adventitia.

In the rabbit (fig. 3) there was practically no intimal staining. Media cells between the elastic membranes showed a positive reaction, more marked in the external layers. Adventitial fibrocytes were also positive.

In the guinea pig (fig. 4) endothelial cells were more markedly stained, particularly in the bottom of tissue folds, along with occasional connective tissue cells in the intima. The media stained weakly, and adventitial fibrocytes stained strongly.

In the golden hamster (fig. 5) there was sometimes a weak positive reaction in the intima (endothelial and connective tissue cells) with an exceedingly weak reaction in the media. Branched fibrocytes in the adventitia reacted strongly.

**Quantitative Differences.** From the microscopic preparations per se it could be seen that the rat aorta reacted much more intensely than the rabbit aorta, while the guinea pig and hamster aorta showed very weak reactions.

After a one hour incubation of paraffin-embedded sections with alpha-naphtol acetate and the diazonium-fluoroborate of 5-chloro-2-toluidine, the amounts of freed alpha-naphtol /cubic mm. aorta in rat and rabbit were 19 ± 4.7 μg. and 4 ± 0.7 μg. respectively.

**Discussion**

Many authors concerned with the question of species susceptibility to atherosclerosis have reported interesting differences in aortic vasa vasorum, or in the mast cells of the vessel wall. Others have found differences in blood chemistry and body metabolism.
It is, however, necessary to state, in concurrence with Holman, McGill, Strong and Geer,\textsuperscript{20} that the vascular wall must be considered as an organ capable of synthesizing, accumulating, and removing lipids, as well as carrying on other metabolic functions.

Our findings on the relationships of aortic enzymatic activity in the species studied, estimated both biochemically (the degree of freeing of unesterified fatty acids from hyperlipemic serum) and histochemically (by means of alpha-naphthol acetate and naphtol AS-acetate) may serve as further evidence concerning the role of these enzyme systems in explaining species susceptibility to experimental as well as to spontaneous atherosclerosis. Deposition of triglycerides in vessel walls may be facilitated by a low enzymatic activity of these tissues.\textsuperscript{1} It must be borne in mind that along with triglycerides, cholesterol may also be deposited, since the latter is partly maintained in solution by the presence of triglycerides.\textsuperscript{21–23}

The biochemical results of lipolytic (lipoproteinolytic) activity do not permit conclusions on the relative importance of individual layers of vessel wall in relation to the deposition of lipids. On the other hand, the histochemical studies of esterase activity have shown that various layers have different degrees of activity, and that there are species differences in the localization of esterase activity in the intima, media, and adventitia. According to present-day concepts, the inner layers of vessel wall, intima, and most of the media are supplied with nutrition including lipids by diffusion from the lumen. Metabolic products and unutilized substances are removed radially in the direction of the adventitia. If this process is interfered with, there may be an accumulation of lipids and other substances, particularly in the intima. Our finding of maximal activity in the media of the rat aorta, with less activity in other species, is in good agreement with the view that a low lipolytic activity in the media leads to a lowering of the concentration gradient, and facilitates deposition in the intima.

Further studies are required for an evaluation of the significance of these findings from the viewpoint of the pathogenesis of vascular disease, particularly atherosclerosis.

**Summary**

Lipolytic (lipoproteinolytic) and esterolytic activity of the aortas of rats and other species less resistant to experimental atherosclerosis, have been compared (rabbit, guinea pig, golden hamster, and cock; in the last-named only lipolytic activity was measured). Nonspecific esterase and AS-esterase were estimated histochemically by an azo-coupling method with the use of alpha-naphthol acetate or naphtol AS-acetate as substrate. Lipolytic activity was estimated biochemically in terms of the amount of unesterified fatty acids freed during incubation with hyperlipemic serum.

Lipolytic activity in the aorta of species susceptible to experimental atherosclerosis is lower than in the aorta of rat, the latter being a very resistant species. The histochemical results were in good agreement with the above, and presented information on the localization of enzymatic activity in individual layers of vessel wall.

**Acknowledgment**

The authors would like to thank Mrs. J. Hájková and Mrs. J. Dvořáková for technical assistance, and Mr. M. Bartoníček for statistical calculations.

**Summario in Interlingua**

Le grados de activitate lipolytic (lipoproteinolytic) e esterolytic del aorta esseva comparate inter rattos e altere species que es minus resistente al induction de atherosclerosis experimental. Iste altere species esseva conilios, porcos de India, hamsters, e gallos. In le gallos, solmente le activitate lipolytic eseva nesurate. Esterase non-specific e AS-esterase eseva estimate histochemicamente per un metodo de azo-copulage con le uso de alpha-naphthol-acetato o naphthol-AS-acetato como substrato. Le activitate lipolytic (lipoproteinolytic) eseva estimate biochimicamente super le base del quantitate de nonesterificate acidos grasse liberate durante le incubation con sero hyperlipemic.

Le activitate lipolytic in le aorta de species susceptible de disveloppar atherosclerosis experimental es plus basse que in le ratto. Rat-
tos es un specie molto resistente. Le risultatos histochimic esseva in bon accordo con supracitate observationes e forniva nove datos relative al localisation del activitate enzymatic in le varie stratos del pariete vascular.

REFERENCES

ERRATA
Page 86 Right column; sentence starting with line 6 should read as follows:
A tracheal cannula was inserted; a femoral vein was cannulated for subsequent intravenous injections and systemic blood pressure was recorded from a common carotid artery connected to a mercury manometer or by a Statham transducer.

Page 90 Left column; sentence starting with line 7 should read as follows:
Since the same concentration (0.003 per cent) was used with both drugs, it appears that these agents are equally effective in raising the threshold of response to electrical stimuli.

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Circ Res. 1959;7:286-290
doi: 10.1161/01.RES.7.3.286

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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