Hydrolase Activities in the Rat Aorta

I. Effects of Diabetes Mellitus and Insulin Treatment

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SUMMARY Vascular disease in diabetics could arise in part from altered vessel wall catabolism. Specific activities of hydrolases in aortic smooth muscle cells from rats with streptozotocin-induced diabetes were measured. Enzymes included: neutral α-glucosidase, α-mannosidase, and lysosomal N-acetyl β-glucosaminidase, β-galactosidase, cathepsin C, acid α-glucosidase, and acid cholesteryl esterase. After 4, 8, and 11 weeks of diabetes, activities of all enzymes studied were decreased significantly in diabetic vessels, decreases ranging from 15% for cathepsin C to 62% for α-mannosidase. After 3 weeks of diabetes, insulin treatment for 1 week restored enzyme levels to normal. After 7 weeks of diabetes, 1 week of insulin treatment did not restore enzyme levels fully to normal (acid cholesteryl esterase was unchanged); 4 weeks of insulin did. Acid phosphatase and N-acetyl β-glucosaminidase activities were reduced markedly in histochemical studies of diabetic aortas at all time periods and were restored by insulin treatment. Alloxan-induced diabetes gave results similar to those with streptozotocin. Significant decreases of aortic hydrolase activities, including those of lysosomes, occur in experimental diabetes mellitus and could contribute to accumulation of substrates in vascular smooth muscle cells.

EPIDEMIOLOGICAL studies in man have identified diabetes mellitus as one of the risk factors associated with atherosclerosis.1,2 Although an occasional study has focused on the contribution of vessel cell metabolism to this association,3 most emphasis has been given to changes in circulating lipoprotein patterns in diabetes mellitus.1,2,4 A vasculopathy occurs in diabetes mellitus which is characterized by basement membrane thickening in small arteries and arterioles, particularly those of the kidney. Although a genetic basis for this thickening has been proposed, its occurrence in normal kidneys transplanted into diabetic recipients suggests that it may be acquired.5 Based on findings of increased carbohydrate of kidney basement membrane in diabetic man8 and increased levels of the synthetic enzyme, glucosyltransferase, in kidneys of animals with experimental diabetes mellitus, Spiro and Spiro9 proposed that the basement membrane accumulation reflects increased synthesis when hyperglycemia is present. In addition to biosynthetic studies, investigations of the catabolic machinery in the vessel wall may provide insights into the pathogenesis of vascular disease. For example, lysosomes are affected markedly in atherosclerotic and hypertensive vascular diseases,8,9,10 and de Duve11 has proposed that lipid accumulation in atherosclerosis may reflect a subtle form of storage disease due to relative deficiency of acid lipase activity in the lysosome. The present studies were carried out to examine the effects of experimental diabetes mellitus on aortic hydrolase activities and to determine how these changes might be related to both the accelerated atherosclerosis and diabetic vasculopathy found in man.

Methods

Male Sprague-Dawley rats (Marland Farms), age 6 weeks and weighing 175-200 g at the outset, were injected with either 10 mg of streptozotocin (Streptozocin; Upjohn) (approximately 55 mg/kg body weight) dissolved in 0.5 ml of 0.9% saline with 0.02 M sodium citrate, pH 4.5, or with 6.7 mg of alloxan (alloxan monohydrate; Sigma) (approximately 40 mg/kg body weight) dissolved in 0.5 ml...
of normal saline. All injections were made via the jugular vein while the rats were lightly anesthetized with ether; controls also were anesthetized for ear marking. Rats given streptozotocin (SZ) ate ad libitum before and after injection; subsequent mortality was 1–2%. Alloxan-injected rats (AL) were fasted for 24 hours prior to injection. Alloxan was injected in the late afternoon so that the early period of profound hypoglycemia known to occur after this dose coincided with the period of heavy feeding at night; even so, an approximate 20% mortality routinely occurred during the first 1–2 days. Overt diabetes mellitus ensued 3 days after injection of streptozotocin or alloxan, as manifested by increased water and food intake and increased urine excretion associated with poor weight gain. Serum glucose concentrations, measured by the glucose oxidase method on a Beckman glucose analyzer (Beckman Instruments) were elevated uniformly several days after injection of the diabetogenic agent.

These values in blood, obtained by tail bleeding from nonfasted rats in midmorning, were remarkably stable over the experimental period (Table 1). Randomly obtained blood samples from diabetic rats always contained more than 250 mg/dl of glucose when tested with glucose oxidase reagent strips (Dextrostix; Ames). Acetonuria (Acetest; Ames) was uniformly absent in rats with streptozotocin-induced diabetes. In general, alloxan-induced diabetes was more severe than that produced by streptozotocin as judged by serum glucose levels and mortality rates.

Insulin-treated rats were injected subcutaneously with 6 U of a long-acting insulin (Lente insulin; Iletin U-40; Eli Lilly) daily in midmorning for the 1-week injection period (see below) or daily in late afternoon for the 1-month injection period (see below). Response to insulin treatment was manifested by improved appearance of the rats (less scruffiness and soiling) and return of the intake and excretion patterns to normal. Restoration of serum glucose concentrations to normal (and frequently somewhat below normal) was documented at least once when the insulin treatment period was a week and at least twice when the treatment period was a month in duration. Blood samples for glucose levels always were obtained 3 hours after the insulin injection, whether in midmorning or later afternoon.

**Experimental Designs**

**Four-Week SZ Experiment**

There were three groups: nondiabetic controls, streptozotocin-injected rats with documented diabetes for 4 weeks and streptozotocin-injected rats, diabetic for 3 weeks and treated with daily insulin for 1 week.

**Four-Week AL Experiment**

This was the same as the previous experiment except that the diabetogenic agent was alloxan monohydrate.

**Eight-Week SZ Experiment**

Three groups again were studied, controls, streptozotocin-injected diabetics, and insulin-treated diabetics. The duration of diabetes was 8 weeks and the insulin-treated group was given its insulin daily for 1 week following a 7-week period of diabetes.

** Eleven-Week SZ Experiment**

In this instance, the experimental period was 11 weeks for three groups: controls, streptozotocin-injected diabetics, and insulin-treated diabetics. The latter group was

**Table 1 Characteristics of Animal Groups Studied**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin-treated diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td>(mg/dl)</td>
<td>(µg)</td>
</tr>
<tr>
<td>4 Weeks streptozotocin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body weight</td>
<td>350 ± 31 (24)</td>
<td>243 ± 41 (28)</td>
<td>250 ± 28 (24)</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>134 ± 9 (24)</td>
<td>531 ± 65 (28)</td>
<td>79 ± 34 (24)</td>
</tr>
<tr>
<td>Total DNA/4 aortas</td>
<td>174.3 ± 25 (6)</td>
<td>148.3 ± 14 (7)</td>
<td>140.8 ± 15 (6)</td>
</tr>
<tr>
<td>4 Weeks alloxan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body weight</td>
<td>344 ± 36 (24)</td>
<td>233 ± 42 (24)</td>
<td>240 ± 34 (24)</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>126 ± 10 (24)</td>
<td>611 ± 116 (24)</td>
<td>90 ± 42 (24)</td>
</tr>
<tr>
<td>Total DNA/4 aortas</td>
<td>164 ± 18 (6)</td>
<td>176 ± 18 (6)</td>
<td>172 ± 11 (6)</td>
</tr>
<tr>
<td>8 Weeks streptozotocin</td>
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<td></td>
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<tr>
<td>Final body weight</td>
<td>408 ± 32 (24)</td>
<td>270 ± 52 (28)</td>
<td>256 ± 29 (24)</td>
</tr>
<tr>
<td>Blood glucose</td>
<td>141 ± 13 (24)</td>
<td>570 ± 90 (24)</td>
<td>63 ± 15 (24)</td>
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<tr>
<td>Total DNA/4 aortas</td>
<td>157 ± 8 (6)</td>
<td>149 ± 21 (7)</td>
<td>136 ± 27 (6)</td>
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<tr>
<td>11 Weeks streptozotocin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body weight</td>
<td>472 ± 52 (24)</td>
<td>309 ± 69 (20)</td>
<td>372 ± 51 (24)</td>
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<tr>
<td>Blood glucose</td>
<td>127 ± 13 (24)</td>
<td>553 ± 118 (20)</td>
<td>57 ± 16 (24)</td>
</tr>
<tr>
<td>Total DNA/4 aortas</td>
<td>215 ± 16 (6)</td>
<td>202 ± 19 (5)</td>
<td>206 ± 7 (6)</td>
</tr>
<tr>
<td>Mature: 4 Weeks streptozotocin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body weight</td>
<td>551 ± 33 (24)</td>
<td>396 ± 32 (20)</td>
<td></td>
</tr>
<tr>
<td>Blood glucose</td>
<td>144 ± 13 (24)</td>
<td>537 ± 61 (20)</td>
<td></td>
</tr>
<tr>
<td>Total DNA/4 aortas</td>
<td>273 ± 18 (6)</td>
<td>246 ± 33 (5)</td>
<td></td>
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</tbody>
</table>

Results are expressed as mean ± sd. Numbers in parentheses = number of animals or groups.
given insulin injections daily for 4 weeks following an initial 7-week period of diabetes.

Four-Week SZ Experiment in Older Rats

Two groups of 14-week-old males, weighing 450–500 g, were used. One group was made diabetic by injection of 20 mg of streptozotocin (approximately 55 mg/kg body weight); the other group served as controls. Early mortality in these rats was approximately 25%, much higher than that found in younger diabetic rats. Surviving diabetic rats were compared to controls after 4 weeks of diabetes.

Morphology and Cytochemistry

One-millimeter rings of ascending aorta were removed from rats which had been anesthetized with ether and exsanguinated via the abdominal aorta, and were fixed at 4°C in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 hours. Rings were rinsed in cacodylate buffer with 7.5% sucrose for 24 hours. For cytochemical studies, sections were incubated for acid phosphatase and N-acetyl-β-glucosaminidase activities, as previously described,15 and examined by light microscopy. Unincubated samples and sections incubated for acid phosphatase activity were postfixed in osmium, embedded, and examined by electron microscopy.12

Biochemical Studies

The segment of aorta between the left subclavian and celiac arteries was quickly removed, opened, and rinsed in sucrose-EDTA solution (0.25 M sucrose-1 mM EDTA). Intima-media strips of the segment were quickly separated from the adventitia13 and placed in the sucrose-EDTA solution at 4°C. Strips of segments from four rats were pooled and suspended in a final volume of 6 ml sucrose-EDTA to make each experimental sample. Five to 7 samples were prepared for each experimental group studied.

Tissue suspensions were homogenized with a motor-driven Teflon pestle in a Potter tissue homogenizer (Arthur H. Thomas Co.). Homogenization with 15 strokes in 4 minutes at 4°C routinely gave a well-dispersed homogenate. Two milliliters of homogenate were used for DNA determinations (see below) and the remainder was divided into 2-ml samples and frozen at −20°C for subsequent enzyme assays.

Enzyme Assays*

Glycosidases and cathepsin C were assayed fluorometrically, as described by Peters et al.,16 except that the following incubation conditions were used:

Neutral α-glucosidase: 1 mM 4-methyl umbelliferyl-α-D-glucopyranoside, 50 mM Na phosphate buffer, pH 6.5, and 0.1% Triton X-100.

α-Mannosidase: 1 mM 4-methyl umbelliferyl-α-D-mannopyranoside, 50 mM Na phosphate buffer, pH 5.9, and 0.1% Triton X-100.

β-Galactosidase: 0.25 mM 4-methyl umbelliferyl-β-D-galactopyranoside monohydrate, 50 mM Na citrate buffer pH 3.6 (for SZ) or 3.9 (for AL), 5 mM MgCl₂, and 0.1% Triton X-100.

Cathepsin C: 0.5 mM glycy1-l-phenylalanyl-β-naphthylamide, 50 mM Na acetate buffer, pH 4.2, 40 mM NaCl, 6 mM dithiothreitol, and 0.1% Triton X-100.

N-Acetyl-β-glycosaminidase: 0.25 mM 4-methyl umbelliferyl-2-acetamido-2-deoxy-β-D-glycopyranoside, 50 mM Na citrate buffer, pH 4.5, and 0.1% Triton X-100.

Acid α-glucosidase: 1 mM 4-methyl umbelliferyl-α-D-glucopyranoside, 50 mM Na acetate buffer, pH 3.9, and 0.1% Triton X-100.

After the addition of 0.1 ml of substrate mixture to 0.1 ml of appropriately diluted enzyme, incubations were carried out for 30 minutes to 5 hours at 37°C, depending on the enzyme studied.

Acid cholesteryl esterase was determined by a modification of the radioisotopic methods of Brecher et al.16 and Takano et al.,17 as described previously,16 except that the final substrate solution contained 3.0 mM Na taurocholate and 100 mM Na acetate buffer, pH 4.2, conditions found to be optimum for the rat aortic activity. The reaction was begun by addition of 0.1 ml of this mixture to 0.1 ml of suitably diluted enzyme. After incubation for up to 5 hours at 37°C, the reaction was stopped and the released radioactive fatty acid was extracted by the procedure of Belfrage and Vaughan.19 Details of this procedure on aortic tissue have been presented elsewhere.18

Under the conditions described, all enzyme activities were linear with time and protein concentration. In each experiment, all enzymes were measured at two dilutions of the homogenate to provide an internal control of linearity of activity. Enzyme activities are expressed in milliunits. One milliunit of activity is defined as 1 nm of substrate hydrolyzed per minute at 37°C. Statistical analysis of results was done with Student's non-paired t-test.

Analytical Procedures

Protein was determined by the method of Lowry et al.20 after prior solubilization of tissue with 0.5% sodium dodecyl sulfate in 0.1 N NaOH at 37°C for 1 hour and using bovine serum albumin as standard. DNA was measured by the method of Burton21 using d-ribose and calf thymus DNA as standards.

Materials

All chemicals were of analytical grade. Sucrose was purchased from Fisher Scientific Co. The 4-methyl umbelliferyl glycoside substrates were purchased from Koch Light Laboratories, Ltd. Glycy1-l-phenylalanyl-β-naphthylamide was purchased from Vega-Fox Biochemicals. Cholesteryl oleate was purchased from Applied Science Labs, Inc., and egg lecithin was from Lipid Products. Most other biochemicals were obtained from Sigma Chemical Co.; cholesteryl 14C-oleate (specific activity 50 mCi/mmol) and 14C-oleic acid (specific activity, 50.6 mCi/mmol) were provided by New England Nuclear. Glutaraldehyde and osmic acid were purchased from Polysciences, Inc.
Results

Characteristics of Animal Groups

Final body weights, blood glucose levels, and DNA content per pooled sample of four aortas are shown for each experiment in Table 1. In every experiment, diabetic rats weighed less than age-matched controls (P < 0.001). When insulin treatment was given for 1 week, body weights of treated rats did not differ significantly from those of untreated diabetics. Only in the 11-week streptozotocin experiment, in which insulin was given for 4 weeks (Table 1), did these diabetic rats attain final body weights greater than those of untreated diabetics (P < 0.01), although still less than controls (P < 0.001).

In every experiment, blood glucose levels in diabetic groups were greater (P < 0.001) than levels in controls (Table 1). When insulin was given, glucose levels in treated groups were always less than those in either diabetic or control groups (P < 0.001 for both comparisons in all groups).

Measurements of total DNA per pooled sample showed no significant differences among control, diabetic, and insulin-treated values in any experiment except for the 4-week streptozotocin experiment. In that instance, DNA values of both treated and untreated diabetic groups were less than control values by about 15% (P < 0.05 for control vs. diabetic and P < 0.02 for control vs. insulin-treated diabetics) but did not differ from each other.

It is noteworthy that rats made diabetic while young (6 weeks) continued to gain weight steadily from initial weights of 175-200 g, but at a much reduced rate compared to controls (Table 1; compare 4-week, 8-week, and 11-week diabetic animals). On the other hand, rats made diabetic at age 14 weeks showed significant decreases in weight from prediabetic levels (Table 1).

In every experiment, abdominal fat was virtually absent in diabetic rats, whatever the age of onset or duration of diabetes. Abdominal fat depots reappeared in insulin-treated rats.

Biochemical Findings

A summary of the kinetic behavior of all enzymes studied from control and streptozotocin-diabetic vessels is shown in Figure 1. A linear relationship between substrate hydrolyzed and protein-time product was found for each enzyme studied. Differences between control and diabetic activities based on DNA (described below) were already apparent during the kinetic studies which used protein as the reference base. Similar plots were obtained for the four hydrolases studied in aortas from rats with alloxan-induced diabetes. Data for aortas from insulin-treated rats have been omitted for the sake of clarity on the figure but were linear and fell close to those from control vessels.

Four-week SZ and AL Experiments

Figure 2 shows the biochemical findings in pooled aortas of the groups in which streptozotocin was the diabetogenic agent. Specific activity of each hydrolytic enzyme studied was less in diabetic vessels than in controls. The percentage of control activity ranged from 46% for N-acetyl-β-glucosaminidase to 79% for cathepsin C; acid cholesteryl hydrolyase activity was 75% of the control value (Fig. 2). In every instance but one, specific activities of aortic hydrolases in insulin-treated diabetic rats were not significantly different from control levels, ranging from 90% to 100%; β-galactosidase reached only 80% of the control value.

Figure 3 shows specific activities of four aortic hydrolases when alloxan was the diabetogenic agent. Diabetic values again were consistently less than controls. The percentage of control activity ranged from 39% for α-mannosidase to 63% for cathepsin C. Levels of enzymes in insulin-treated rats did not return as close to control values as they had in the streptozotocin diabetes experiment. Three of the four enzymes returned to intermediate levels, ranging from 68% of the control value for neutral α-glucosidase to 79% of the control value for α-mannosidase. In contrast, cathepsin C achieved a level (91% of control) which was not significantly different from control.

Mixing experiments were carried out to examine the possibility that an enzyme inhibitor might be present in homogenates of diabetic aortas which could account for
AORTIC HYDROLASES IN DIABETES MELLITUS/Wolinsky et al.

Figure 2 Hydrolyase activities in aortic homogenates of 4-week streptozotocin experiment. Specific activities of hydrolases, expressed per milligram DNA, are shown for control, diabetic, and insulin-treated groups. Duration of streptozotocin diabetes was 4 weeks, with the I group given insulin for the final week. Individual data points are presented for each group, together with lines representing calculated mean and standard deviations. Statistical significances of differences between groups are given at the base of each panel.

Figure 3 Hydrolyase activities in aortic homogenates of 4-week alloxan experiment. Duration of alloxan diabetes was 4 weeks with the I group given insulin for the final week. Refer to legend to Figure 2 for further details.

Table 2 shows N-acetyl-β-glucosaminidase specific activities after mixing different proportions of homogenates from control and streptozotocin-diabetic rats. Activities observed in two such mixing experiments fell close to those predicted mathematically from the original pure control and diabetic homogenates. The same results were obtained from mixing experiments carried out using alloxan-diabetic tissue. The striking similarity in enzyme response to alloxan or streptozotocin injections thus permitted us to study the effect of only streptozotocin in subsequent long-term experiments. The latter had the obvious advantage of decreased mortality of experimental rats.

Table 2 Enzyme Activity of Mixed Control and Diabetic Aortic Homogenates

<table>
<thead>
<tr>
<th>Proportions*</th>
<th>NAGA† activity (mut/mg DNA)</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Diabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>0.75</td>
<td>44.93</td>
<td>36.06</td>
</tr>
<tr>
<td>0.25</td>
<td>0.75</td>
<td>53.80</td>
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<tr>
<td>0.75</td>
<td>0.25</td>
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</tr>
<tr>
<td>0.75</td>
<td>0.25</td>
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<td>67.85</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>81.94</td>
<td>71.53</td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td>95.72</td>
<td>83.15</td>
</tr>
</tbody>
</table>

* Two separate experiments are shown here. Stated proportions of aortic homogenates from control and streptozotocin-diabetic rats were mixed to a final volume of 0.1 ml and incubated with 0.1 ml of substrate. The activities of different mixtures were compared with those calculated from assays performed on pure diabetic and pure control homogenates. All observed activities were within the linear range of the assay.
† NAGA = N-acetyl-β-glucosaminidase.
‡ nmol substrate hydrolyzed/min.
Eight- and 11-Week SZ Experiments

The significant and marked decreases in all hydrolase activities observed at 4 weeks persisted after 8 weeks of diabetes (Fig. 4). The percentage of control activity remaining in diabetic vessels for each enzyme was remarkably similar to that found after 4 weeks of diabetes for five of seven hydrolases studied. Cathepsin C activity was 79% of control at 4 weeks and 61% of control at 8 weeks, and acid cholesteryl esterase activity which had been 75% of control at 4 weeks was 84% of control at 8 weeks. Comparison of the insulin-treated group with untreated diabetics showed an increase in activities of all but one hydrolase; only acid cholesteryl esterase activity was unchanged (83% of control). Of the six hydrolases affected by insulin treatment, only α-mannosidase and cathepsin C reached control levels (91% and 102%, respectively). The responses of the other four were at intermediate levels, ranging from 77% of control values for N-acetyl-β-glycosaminidase to 86% of control values for neutral α-glucosidase.

Results of the 11-week experiment show differences from those at 8 weeks. Although enzyme activities in diabetic vessels from 11-week experimental rats generally were comparable to those in the 8-week experiment, when considered as a percentage of control values (Fig. 5), there were noted differences in the insulin-treated group. In contrast to the 8-week experiment, in which 1 week of

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**FIGURE 4** Hydrolase activities in aortic homogenates of 8-week streptozotocin experiment. Duration of streptozotocin diabetes was 8 weeks with insulin given to the 1 group for the final week. Other details in legend to Figure 2.

**FIGURE 5** Hydrolase activities in aortic homogenates of 11-week streptozotocin experiment. Duration of diabetes was 11 weeks with 1 group given insulin for the last 4 weeks. Other details are described in the legend to Figure 2.
insulin treatment resulted in restoration of only two hydrolases to near-control activity, 4 weeks of insulin treatment in the 11-week experiment resulted in restoration to control levels of all hydrolases except /3-galactosidase. This enzyme reached 85% of the control level, an intermediate range for that enzyme as found in insulin-treated rats in each of the previous experiments. Interestingly, cathepsin C levels in insulin-treated rats reached 124% of control levels after 4 weeks of insulin treatment (P < 0.02). Acid cholesteryl esterase activity, which was unaffected by 1 week of insulin treatment (Fig. 4), was restored to the control level after 4 weeks of insulin.

**Mature Rat: 4-Week Streptozotocin Experiment (Not Shown)**

Vessels from rats made diabetic with streptozotocin at 14 weeks of age were assayed after 4 weeks of diabetes for their patterns of hydrolytic enzymes. Specific activities of enzymes in older controls were entirely comparable to those found in younger controls. Furthermore, specific activities of all enzymes studied in diabetic vessels were significantly lower than those in controls, ranging from 23% to 79% of control levels, values similar to or slightly lower than those of younger diabetics. These observations are noteworthy since older rats were growing at a rate approximately one-fifth that of younger animals (Table 1).

**Morphological Findings**

Grossly, vessels removed from diabetics tended to be thinner than those from controls. This difference was most noticeable after 4 weeks of diabetes and diminished as the duration of diabetes increased, although it persisted in all groups, including the mature diabetics.

Cytochemical results were very similar at all periods, whether the diabetogenic agent used was streptozotocin or alloxan. In controls, incubation of aortic slices for acid phosphatase or N-acetyl-/?-glucosaminidase activity showed the usual sparse number of reactive lysosomes seen in young rats.9 In vessels from diabetics of either the alloxan type (Fig. 6B) or the streptozotocin type (Fig. 6C), many fewer reactive lysosomes were seen with either enzyme substrate; they were, in fact, difficult to find. In vessels from insulin-treated rats (Fig. 6D), the apparent number of lysosomes was similar to that of control vessels; occasionally, even more than the normal number seemed to be present.

Electron microscopic examination of tissues incubated for acid phosphatase activity confirmed the localization of enzyme reaction product in lysosomes and were consistent with the light microscopic findings in the three groups of rats studied. In the youngest age group, acid phosphatase reaction product was also seen in the parallel saccules of the Golgi apparatus and Golgi-associated vesicles (Fig. 7, upper panel). The most common type of lysosome was the dense body. Autophagic vacuoles rarely were seen.

Electron microscopy did not reveal any structural abnormalities or evidence of cellular damage or necrosis in the experimental groups. The morphological hallmarks of aortic smooth muscle cells, including surface pinocytic vesicles, myofilaments, abundant mitochondria, dilated rough endoplasmic reticulum and, in the younger rats, a prominent Golgi apparatus, were similar in the control, diabetic, and insulin-treated rats.

The increase in cell size and proportion of the cytoplasm occupied by myofilaments associated with maturation also occurred in all three groups. In the 11-week experiment, the Golgi apparatus was less prominent and lysosomes with small lipid-like inclusions were more common (Fig. 7, lower panel).

**Discussion**

Although our primary interest was in lysosomes, other enzymes studied here probably are located in other cellular compartments. Neutral a-glucosidase is considered to be a microsomal marker in aortic tissue, based on analytical cell fractionation studies of rabbit15 and calf16 aortic smooth muscle cells. The pH optimum of 6.5 and the specific activity of this enzyme in rat aortic cells agree well with data obtained from normal rabbit15 and calf16 aortic cells. The pH optimum of the major a-mannosidase activity found in rat aortic cells agrees best with the Golgi-associated enzyme described by Dewald and Touster.29 Although definite proof of such localization of this enzyme is not possible from our studies, the presence of a small shoulder of activity in our homogenates at pH 4.5 (published data) seems to suggest that the major activity measured at pH 5.9 is not from lysosomes. The activities of /3-galactosidase, cathepsin C, N-acetyl-/?-glucosaminidase, and acid a-glucosidase are lysosomal. All activities showed acid pH optima and had specific activities in the rat aortic homogenates comparable to those found in smooth muscle cells isolated from normal rabbit15 and calf16 aortas.

The cholesteryl ester hydrolase studied here had an acid pH optimum of 4.2 and showed activation by sodium taurocholate and a requirement for Triton as has previously been described for the lysosomal enzyme in aortic smooth muscle cells.18 The specific activity of the enzyme in rat aorta, however, was markedly lower than the levels found in normal rabbit and calf aortic cells; the reasons for this, other than inherent species differences, are not known.

The studies reported here indicate that the activities of many hydrolases of the vessel wall, including those found in lysosomes, are reduced in diabetic vessels. These decreases generally persist for at least 11 weeks, may become progressively more severe, and do not reflect growth rate. We cannot rule out the possibility that these decreases reflect relative starvation of the rats in the face of marked insulin deficiency. Insulin treatment reverses the enzyme decreases to varying degrees, depending on the particular enzyme and durations of the prior diabetic and effective insulin treatment periods. Cytochemical studies support these biochemical findings. No obvious ultrastructural alterations other than those related to lysosomes are seen in vessels from diabetic or insulin-treated animals.

There has been little previous work on hydrolytic and catabolic processes in vascular tissue of diabetics. In a
Aortic lysosomes in streptozotocin- and alloxan-induced diabetes; 4-week experiments. Light microscopy of sections of rat thoracic aorta incubated 90 minutes for N-acetyl-β-glucosaminidase activity: A = control, B = alloxan-induced diabetic, C = streptozotocin diabetic, D = insulin-treated streptozotocin diabetic. Reactive lysosomes (arrows) are numerous in the control vessel (A), markedly diminished in the diabetics (B and C), and are again numerous in the insulin-treated diabetic (D). X1600.

series of studies on diabetic rats, Fushimi and Tarui reported decreased acid glycosidase levels in kidney tissue which they attributed to the renal vascular component. Enzyme levels could be restored toward normal by insulin administration for 3 days after 8 weeks of diabetes. These authors stressed the possible role of decreased catabolism of glycoproteins in the genesis of the renal microangiopathy of diabetes. Recently, Nagakawa et al. reported decreased specific activities of lysosomal β-hexosaminidase and α-glucosidase in skin fibroblast cultures from patients with diabetes mellitus compared to age-matched controls; these differences were not found in leukocytes.

In contrast to the decreases found in tissue levels of hydrolytic enzymes, several studies have reported that activities of synthetic enzymes in diabetic tissues are increased or unchanged. In diabetic rat kidneys, Spiro and Spiro found increased levels of a glucosyltransferase which is involved in basement membrane synthesis. Their studies of several other tissues (not including aorta) from diabetic animals showed levels of this synthetase to be unchanged from those of normal animals. Aortic levels of this enzyme also appear to be unchanged in rats with streptozotocin-induced diabetes of 8 weeks' duration (A.M. Adamany and H. Wolinsky, unpublished observations). Beisswenger and Spiro have proposed that the increased carbohydration of glomerular basement membrane from human diabetics might be related to increased
**Figure 7** Upper panel: electron micrograph of aortic smooth muscle cell incubated for acid phosphatase activity from control animal; 8-week experiment. Note the characteristic myofilaments (my) and pinocytic vesicles (p). Dense enzyme reaction product (lead phosphate) is seen as black accumulations over lysosomes (ly) and Golgi saccules and vesicles (G). The scale bar equals 1 μm. Lower panel: aortic smooth muscle cell from rat with streptozotocin-induced diabetes; 11-week experiment. Lysosomes (ly) with lipid-like inclusions (arrow) are more numerous in this age group. The scale bar equals 0.5 μm.
thickening of basement membrane, which leads to the typical microangiopathy. Others have found increased activity of lysyl hydroxylase in diabetic kidneys. Both the glycosyltransferase and lysyl hydroxylase activities decreased toward normal levels after insulin administration. Although no direct connection between the different effects of diabetes on synthetic and catabolic activities of vascular smooth muscle cells currently exists, it is tempting to speculate that they are related and that the net result is accumulation of glycoproteins in the vessel wall.

The findings reported here also may be relevant to atherosclerosis, the macroangiopathy associated with diabetes mellitus. Increased levels of circulating lipoproteins are found in diabetic humans and experimental animals. However, in epidemiological studies, such elevations cannot account totally for the enhanced clinical atherosclerosis found in diabetics. The present findings show that the specific activity of aortic acid cholesteryl hydrolase, a lysosomal enzyme involved in lipoprotein catabolism, is decreased despite increased levels of cholesterol-ester rich lipoproteins in this animal model. De Duve has postulated that cholesterol ester accumulation in atherosclerotic vessels could reflect a relative deficiency of acid lipase within vascular smooth muscle cells. In the presence of high levels of substrate (cholesterol ester-rich lipoprotein), incomplete hydrolysis would lead to progressive intralysosomal accumulations of cholesterol ester and development of vascular disease. This hypothesis has found support from experimental animal models in which lipid levels were raised by cholesterol feeding and from studies of experimental hypertension. Permeability of diabetic vessels to cholesterol is different from that of controls in that the amount of influx seems to be related solely to the circulating cholesterol level. In diabetes mellitus, the coexistence of normal or, more likely, increased influx of lipids into the vessel wall and decreased clearance by the lysosomal lipase creates a setting in which accelerated atherosclerosis might occur. Further studies are needed to determine the long-term effects of these hydrolyase deficiencies on accumulations of glycoproteins and lipids in the vessel wall and the cellular consequences of interactions of diabetes mellitus with other clinical risk factors.

Finally, we wish to emphasize that integration of currently available information from studies on vascular metabolism in diabetes mellitus is clearly impossible. Alterations in lipid synthesis and oxidative and nonoxidative metabolism of glucose in vascular tissue have been described. Elevations in plasma and urinary lysosomal enzymes have been found in diabetic man and experimental animals. Furthermore, many hormonal derangements in human and experimental diabetes, in addition to insulin lack, are present, any one of which, including hyperglycemia itself, could be responsible for the observed results. In fact, experimental diabetes mellitus of the type studied here, with severe insulin deficiency, glucagon excess, and absent ketosis, is far from an exact counterpart of the more complicated metabolic derangement found in human diabetes mellitus. The present study has demonstrated altered vascular smooth muscle cell catabolic activity in experimental diabetes mellitus. The importance of this component in the complex pathogenesis of accelerated vascular disease in clinical diabetes mellitus remains to be determined.

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Hydrolase Activities in the Rat Aorta

II. Effects of Hypertension Alone and in Combination with Diabetes Mellitus

HARVEY WOLINSKY, LOIC CAPRON, SIDNEY GOLDFISHER, FREDERIQUE CAPRON, BERNICE COLTOFF-SCHILLER, AND LISA E. KASAK

SUMMARY Hypertension is an important risk factor for atherosclerosis and often occurs in association with diabetes mellitus. Specific activities of hydrolases in homogenates of aortas from rats with renal-clip hypertension, normotension following a period of hypertension, and hypertension combined with streptozotocin-induced diabetes mellitus were measured. Enzymes included: neutral α-glucosidase, and lysosomal N-acetyl-β-glucosaminidase, β-galactosidase, cathepsin C, acid α-glucosidase, and acid cholesteryl esterase. After 6 or 12 weeks of hypertension, specific activities of all enzymes measured were significantly increased, levels ranging from 24% above normal for cathepsin C to 351% above normal for N-acetyl-β-glucosaminidase. Six weeks of normotension following 6 weeks of hypertension resulted in restoration to normal of four of the six enzyme activities; the remaining two enzymes were significantly below normal levels. Combined hypertension and diabetes mellitus showed smooth muscle cell levels of four of the five hydrolase activities to be significantly lower than those present with hypertension alone. In every instance, histochemical studies of aortas showed acid phosphatase and β-N-acetyl-β-glucosaminidase activities which corresponded to the biochemical findings. These findings indicate profound and discrete effects of two clinical risk factors on vascular smooth muscle cell lysosomes.

HYPERTENSION is a major risk factor for the development of atherosclerosis and its sequelae in man.1 Increased vascular permeability has been invoked as a mechanism for this effect of hypertension, since experimental hypertension results in increased permeability of the vessel wall to many circulating proteins and cholesterol.2-3 Presumably, this is reflected in enhanced cellular uptake of circulating materials and stimulation of lysosomes in vascular smooth muscle cells which occurs under these conditions.4-5

Diabetes mellitus is another risk factor for atherosclerosis in man6 which often occurs in association with hypertension.6-8 Permeability of the diabetic vessel wall is reported to be normal6 or increased.10 Yet, in experimental diabetes mellitus, levels of hydrolases, including those in lysosomes, are decreased in vascular smooth muscle cells as shown in the accompanying paper.11

When more than one risk factor is present, each makes an independent contribution to the clinical risk of disease.1 The difference we have observed between the lysosomal responses to two common risk factors, diabetes mellitus and hypertension, therefore was intriguing and stimulated the present studies. Two objectives were sought. First, we wished to extend our previous observations on lysosomal enzymes in hypertension to other hydrolases, including...
Hydrolase activities in the rat aorta. I. Effects of diabetes mellitus and insulin treatment.
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