Effect of Pancreatectomy, with and without Hypophysectomy, and of Insulin Treatment on the Composition of Canine Aorta

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SUMMARY

The chemical composition of aorta from normal and endocrine deficient dogs was studied in terms of collagen, elastin, calcium, "water plus lipid" and mucopolysaccharide (MPS) content. The latter component was resolved into four fractions: hyaluronic acid (HA), heparan sulfate (HS), dermatan sulfate (DS), and the isomeric chondroitin sulfates (CS). The results were compared in the following way: (1) normal versus pancreatectomized; (2) hypophysectomized versus hypophysectomized and pancreatectomized (Houssay); and (3) Houssay with and without insulin treatment. Pancreatectomy affected the content of two constituents only: the HS and CS levels were lowered. The low content could be raised by insulin treatment. These results indicate that except for the sulfated MPS, insulin does not appreciably alter the composition of the aortic wall. DIABETES *22*:397-402, May, 1973.

In the course of a systematic study of the effects of hormones on the composition of the aortic wall, we have reported that hypophysectomy and replacement therapy with either growth hormone (GH) or thyroxine (T_4) caused marked alterations in the content of elastin, deoxyribonucleic acid (DNA) and of mucopolysaccharides (MPS).[1-3] We have now investigated the effect of insulin on aortic tissue. Hormonal deficiency was achieved by pancreatectomy or by pancreatectomy and hypophysectomy, and was partially corrected by replacement therapy with insulin. The results indicated that in comparison with T_4 and GH, insulin has only a weak effect on the composition of the aortic wall.

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MATERIALS AND METHODS

Mongrel dogs of either sex, between the ages of six and eighteen months, and weighing between 8 and 12 kg., were used in these experiments. Previous studies from this laboratory have shown that little difference exists in the composition of aorta between the ages of six and eighteen months in mongrel dogs of either sex.[3] Pancreatectomies were carried out under aseptic conditions with pentobarbital (Nembutal) anesthesia (35 mg./kg. b.w.) as described.[4] Subsequently the animals were fed measured amounts of commercial dog food supplemented with raw pancreas. A mixture of crystalline (8 to 12 U.) and Protamine-Zinc insulin (2 to 5 U., Connaught Medical Research Laboratories) was injected subcutaneously at feeding time. On purpose, the animals were poorly controlled and insulin was administered in amounts sufficient to prevent ketonuria only. Consequently, fasting plasma glucose levels fluctuated around 300 mg./100 ml. (ferricyanide method, Technicon AutoAnalyzer) during the entire period of five weeks, after which the dogs were sacrificed. Using insulin and diet for prolonged periods of time, we were unable to keep diabetic animals normoglycemic and at the same time avoid hypoglycemic episodes. We, therefore, abandoned the idea of maintaining our animals under varied degrees of control. We felt that a comparison of poorly controlled diabetic dogs with normal healthy dogs was more meaningful than a comparison of poorly controlled diabetic animals with less poorly controlled diabetic animals.

Hypophysectomy was performed by the transbuccal technic,[5] and subsequently the animals were maintained for an average of five months without replacement therapy.[6] The Houssay animals were maintained without hormonal substitution on a high protein diet for an average of three months.[6] Fasting plasma glucose levels fluctuated around 300 mg./100 ml., but the animals were not ketonuric. In this respect they resembled

the poorly controlled pancreatectomized dogs. Three of the Houssay dogs received 0.1 U./dog/day of short-acting crystalline insulin subcutaneously for three weeks prior to sacrifice. Fasting plasma glucose levels of these insulin-treated dogs varied between 80 and 100 mg./100 ml. The remaining four Houssay dogs were maintained as controls without substitution therapy.

Animals were sacrificed with an overdose of Nembutal. Aortas were immediately removed, cleaned of adventitia and the whole vessel (from the arch to the iliac bifurcation) was weighed on an analytical balance and then minced and defatted with acetone-ether. Tissues were air-dried and weighed again; the difference between the wet and dry fat-free weight was taken as the "water plus lipid" content of the vessel.

Collagen was estimated as hydroxyproline by the method of Neuman and Logan[7] with the modification of Woessner.[8] The collagen content was calculated by assuming a hydroxyproline content of 13.4 per cent. In a separate study the proline/hydroxyproline ratio was found unchanged in aortas of endocrine deficient dogs.[3] Thus, any differences in hydroxyproline content found under our experimental conditions were true changes in collagen content, and were not the result of altered activity of the hydroxylating enzyme. It is recognized that the conversion factor of 13.4, used here for expressing hydroxyproline values as collagen, is to a certain extent arbitrary, since its validity was not checked under our experimental conditions. Although it might have been more appropriate to express the results as hydroxyproline, the conventional factor was used by us, nevertheless, to facilitate comparison of our data with those from the literature.

Alkali-stable elastin was determined gravimetrically on the residue after collagen was removed, by the method of Lowry, Gilligan and Katersky.[9] Extraction of DNA from tissue was done according to the method of Reich[10] and the DNA content was measured according to Burton.[11] Calcium was measured in a Perkin-Elmer atomic absorption spectrophotometer according to Dawson and Heaton,[12] after extracting the tissue with perchloric acid.

MPS were first released from tissue by digestion with papain according to Schiller, Slover and Dorfman.[13] The MPS were then isolated by alcohol precipitation according to Kaplan and Meyer,[14] and subsequently by precipitation with cetylpyridinium-chloride according to Scott.[15] Total MPS were estimated as uronic acid by the orcinol technic according to Davidson.[16] This fraction was then resolved into individual components by electrophoresis on cellulose acetate strips in 0.2 molar zinc sulfate buffer pH 5.5, 1 mAmp/cm. for ninety minutes according to Breen et al.[17] The strips were stained with Alcian Blue and scanned in a Beckman RB Analytrol. Aortic MPS were identified by comparing their mobilities with standard preparations kindly supplied by Drs. J. A. Cifonelli and L. Roden of the University of Chicago. Heparan sulfate isolated from calf aorta was kindly supplied by Dr. A. A. Horner of this Department. In accord with other work from this laboratory,[2] four distinct fractions were obtained in this system: (1) hyaluronic acid (HA); (2) heparan sulfate (HS); (3) dermatan sulfate (DS); and (4) the isomeric chondroitin sulfates (CS). Each MPS was quantitated by cutting out the appropriate peak on the Beckman Analytrol chart and weighing it on an analytical balance. A linear relationship was obtained for each constituent within the concentration range of 0.5 to 1.5 nanomoles of uronic acid. Repeat analyses of HS, DS and CS either from aorta or standard preparations agreed within 3 per cent, those of HA within 7 per cent.[3]

Results are expressed as the mean ± standard error of the mean. Differences between means were tested for statistical significance using the Student's *t*-test as described by Snedecor.[18] P values of less than 0.05 were taken as significant.

RESULTS

Table 1 gives estimated collagen and elastin contents of aortas from pancreatectomized and from Houssay dogs with and without insulin treatment, and for comparison, also from normal and hypophysectomized dogs.

Collagen represented 23 per cent by weight of the dry defatted tissue of normal whole aorta. This value was not significantly changed in aortas of pancreatectomized dogs. Following hypophysectomy there was a

TABLE 1

Fibrous proteins of whole canine aorta

	Collagen mg./gm. dry defatted tissue (Mean ± S.E.M.)	Elastin
Normal (n = 13)	230 ± 5	333 ± 7
Hypox (n = 12)	250 ± 5*	358 ± 5*
Panx (n = 6)	220 ± 10†	323 ± 11†
Houssay (n = 4)	260 ± 5*	357 ± 7*
Houssay + insulin (n = 3)	273 ± 7*	340 ± 7

* Significantly different from Normal.
† Significantly different from Houssay.

significant increase in the collagen content to 25 per cent, and the figure did not appreciably change after the pancreas was removed from hypophysectomized dogs. Insulin treatment of these Houssay dogs did not normalize the high aortic collagen content.

The elastin content of normal canine aorta represented some 33 per cent by weight, and remained unaltered in pancreatectomized dogs. Hypophysectomy caused a significant increase in elastin content to nearly 36 per cent, and this figure was not altered when hypophysectomy was combined with pancreatectomy. Houssay dogs treated with insulin had a tendency to a lower elastin content, but the difference from untreated Houssay dogs was statistically not significant.

The "water plus lipid" content, the DNA and the total MPS are given in table 2. The "water plus lipid" content was not significantly altered in pancreatectomized animals as compared to normal controls. Both hypophysectomized and Houssay dogs had a slightly lower "water plus lipid" content than did normal controls. Aortas from Houssay dogs treated with insulin were not significantly different from those of untreated Houssay animals.

TABLE 2

Water plus lipid, deoxyribonucleic acid and total mucopolysaccharide in whole canine aorta

	Water + Lipid $\times 10^3$	DNA	Total MPS (uronic acid)
	mg./gm. dry defatted tissue (Mean±S.E.M.)		
Normal (n = 13)	3.02 ± 0.07	5.24 ± 0.16	3.01 ± 0.08
Hypox (n = 12)	2.79 ± 0.08*	6.33 ± 0.21*	1.86 ± 0.04*
Panx (n = 6)	2.88 ± 0.04	5.17 ± 0.12†	2.52 ± 0.10*†
Houssay (n = 4)	2.72 ± 0.06*	6.58 ± 0.16*	1.77 ± 0.06*
Houssay + insulin (n = 3)	2.77 ± 0.12*	5.57 ± 0.15†	2.17 ± 0.10*†

* Significantly different from Normal.
† Significantly different from Houssay.

Whereas hypophysectomy increased the aortic DNA content, pancreatectomy had no effect on this constituent. Likewise, pancreatectomy performed on hypophysectomized dogs did not affect the high DNA content found in aortas of these dogs. However, insulin administration to Houssay dogs caused a significant fall in DNA content, bringing it down to levels seen in normal animals.

The total MPS content was decreased in all types of endocrine deficient dogs. Even though it did not quite normalize it, insulin treatment of Houssay dogs caused a significant increase in total MPS content.

Table 3 gives the distribution of the four mucopolysaccharides studied. While the hyaluronic acid content remained unchanged in aortas of our endocrine deficient dogs, the changes in sulfated MPS were extensive and were responsible for the trends that were already indicated above for total MPS. HS content was decreased after pancreatectomy, hypophysectomy and also in Houssay dogs. Insulin treatment of Houssay dogs raised the aortic HS content somewhat, but not quite to normal values. The DS content was depressed after hypophysectomy. No further reduction was seen if hypophysectomized dogs were also pancreatectomized. Pancreatectomy alone had no significant effect on aortic DS levels, nor did insulin administration to Houssay dogs. Chondroitin sulfate represented over half of the sulfated MPS in normal control aortas. The content was significantly decreased by hypophysectomy and/or pancreatectomy. Insulin treatment of Houssay dogs increased the CS content to a level significantly higher than that in the untreated Houssay dogs but the content still remained below the normal range.

The calcium content of normal control aortas was 11.0 ± 0.3 μmoles/gm. dry defatted tissue. It was not affected by any of the experimental procedures and the values have, therefore, been omitted from tabulation.

TABLE 3

Individual mucopolysaccharides in whole canine aorta

	HA	HS	DS	CS
	Uronic acid mg./gm. dry defatted tissue (Mean ± S.E.M.)			
Control (n = 13)	0.25 ± 0.02	0.56 ± 0.02	0.76 ± 0.02	1.44 ± 0.06
Hypox (n = 12)	0.23 ± 0.02	0.33 ± 0.02*	0.58 ± 0.02*	0.73 ± 0.04*
Panx (n = 6)	0.23 ± 0.02	0.45 ± 0.02*†	0.72 ± 0.02†	1.12 ± 0.06*†
Houssay (n = 4)	0.21 ± 0.02	0.29 ± 0.02*	0.58 ± 0.04*	0.68 ± 0.04*
Houssay + insulin (n = 3)	0.23 ± 0.02	0.40 ± 0.02*†	0.65 ± 0.04*	0.88 ± 0.02*†

* Significantly different from Normal.
† Significantly different from Houssay.

DISCUSSION

In the current series of hypophysectomized dogs the aortic collagen content was significantly higher than that of normal dogs, whereas in an earlier series of experiments[1] this constituent remained within normal limits. This was so in spite of the fact that the urinary excretion of hydroxyproline, the breakdown product of collagen,[19] was greatly diminished.[2,3] The main difference between the two groups is that dogs in the present series were hypophysectomized for an average of five months, whereas those in the former series were hypophysectomized for less than three months. It is possible that the additional length of time after hypophysectomy reduced the breakdown of collagen to such an extent that the net result was an accumulation of this material in aortic tissue. It was only collagen that in our experience behaved in this singular way; the length of time following hypophysectomy did not appear to affect the content of other aortic constituents.

Of the hormones studied so far, the lack of pituitary hormones removed by hypophysectomy and their replacement by GH or T_4 caused a more profound change than the hormonal deficiency produced by pancreatectomy or replacement therapy with insulin. Since we have so far not attempted to replace the loss of pancreatic glucagon, we have no information on its effect on aortic tissue.

There was no change in the content of HA of the aortas of our hypophysectomized, pancreatectomized and Houssay dogs, which is in agreement with the findings of Cohen and Foglia[20] in aortic tissue of partially pancreatectomized rats. In contrast to this, Ichida and Kalant[21] found an increase in aortic HA of untreated alloxan diabetic rats and rabbits. In the skin of insulin-treated alloxan diabetic rats, Schiller and Dorfman[22] observed an increase in the synthesis and in the pool size of HA. These apparently divergent results have one possible explanation in the effect of insulin on the synthesis of HA. Glucose serves as a precursor for both hexosamine and hexuronic acid moieties of this polymer.[23] Consequently, it all depends whether or not the cell is sensitive to insulin. In some cells, where insulin does facilitate glucose transport like in the skin,[24] an increase in HA is to be expected. In other cells, such as aortic tissue, where insulin was shown to have no effect on glucose uptake,[25,26] the HA content would be expected to fluctuate independently.

Aortas of alloxan diabetic rats are known to have a low uronic acid content and diminished radiosulfate uptake, and the defect can be partially remedied by insulin administration.[27,28] Using partially pancreatectomized rats, Cohen and Foglia[20] found a decrease in the concentration of aortic CS in the late stages of diabetes. As was apparent from the uptake of radiosulfate, the reduction in CS content was due to decreased synthesis and increased degradation. In the same study, the turnover of HS and DS was found to be depressed, even though the net aortic content of these MPS remained unchanged. Our own study of the actual content of individual MPS in aortic tissue is not in contradiction with the steady state and turnover data of Cohen and Foglia,[20] with the exception of the HS content, which was decreased under our experimental conditions. However, our data do support their concept that insulin in some way controls the sulfation of MPS.[20,29,30] This is also in agreement with the findings of Ichida and Kalant[21] in alloxanized animals.

Our data provide evidence that the content of collagen and of elastin in canine aorta is not influenced by insulin deficiency or by insulin replacement therapy. So far, there has been no report in the literature on the effect of insulin on connective tissue in vivo in terms of collagen or elastin. In vitro, insulin has been shown to increase collagen synthesis by osteocytes[31] and by granulation tissue.[32] In aortic tissue in vitro, Krahl[33] has observed no effect of insulin on protein synthesis in general.

When the Houssay dogs were treated with small amounts of insulin, the increased concentration of DNA, characteristic for the hypophysectomized state, was brought back to normal. Even though it appears that the reduction in aortic DNA content following treatment of Houssay dogs would be a direct effect of insulin on aortic cells, we do not believe this to be true. If it were so, then normal dogs after pancreatectomy should have an increased concentration of DNA in their aortic tissue, which was not the case. We consider it more likely that the normalization is a secondary effect due to the general improvement of the metabolic condition of these extremely sensitive specimens following insulin administration.

It is extremely difficult to maintain Houssay dogs in a reasonable state of health without multihormonal replacement therapy. Thus, it is inevitable that the number of Houssay dogs suitable for experimentation had to be small. The smallest group was represented by animals treated with insulin ($n = 3$), and the rise in the initially reduced HS and CS content would be difficult indeed to ascribe to the action of insulin, were it not for the fact that a reduction in HS and CS content

was encountered when normal dogs were pancreatectomized. It is this logical interlocking of results from related series of experiments that gave us the confidence to arrive at conclusions in spite of the fact that differences between small groups of animals may be statistically significant, but not necessarily biologically meaningful.

An overall evaluation of the results of our present and previously reported experiments indicates that insulin deficiency and its replacement therapy caused changes in the composition of canine aorta which are much less dramatic, though not necessarily less detrimental, than those we have seen after hypophysectomy and replacement therapy with either GH or T_4.[2] If we may venture to speculate and apply our observations on canine aorta to human pathological conditions, it seems that in the development of diabetic vasculopathies, the relative insulin deficiency is perhaps not the only factor. Indeed, on the basis of our experiments we would be inclined to favor the view that abnormal secretion of other hormones, for example hypersecretion of GH, triggered by anything less than optimal control of the diabetic state,[34] may be an important contributing factor in the development of a diabetic angiopathy.

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