

Effect of Intra-arterial Insulin on Tissue Cholesterol and Fatty Acids in Alloxan-Diabetic Dogs

By ANATOLIO B. CRUZ, JR., M.D., DONALD S. AMATUZIO, M.D.,
FRANCISCO GRANDE, M.D., AND LYLE J. HAY, M.D.

With the technical assistance of Miss Donna E. Swenson, B.S.

DEGENERATIVE vascular disease is a well-known complication of diabetes mellitus and many diabetics die from the resultant damage to the cardiovascular system. Nonfatal illnesses secondary to diabetes mellitus, such as gangrene of the legs, blindness, cerebral vascular accidents and senile mental deterioration, impose a vast morbidity on the diabetic. These, too, are largely accountable to degenerative vascular disease.¹⁻⁵ There are some indications that the incidence of vascular complications in diabetes mellitus has not decreased in spite of the great improvement in the control of the disease.^{4, 6} This may be the result of the increased longevity of the diabetic patient.^{3, 5, 6} Animal experiments have given indications that insulin might have some aggravating influence on the development of atheromatous lesions.⁷⁻¹⁰ It is then possible that the long-term usage of insulin in diabetic patients may play a role in the development of the atherosclerosis in these patients.⁸

The present study was undertaken in an attempt to find out if daily intra-arterial administration of insulin has any effect on the lipid content of the arterial wall in the diabetic animal. The experiments to be reported in-

dicating a significant increase in the fatty acids and cholesterol content of the arteries and muscles of one hind leg of the dog in which insulin was given intra-arterially as compared with the opposite leg injected intra-arterially with normal saline.

Methods

A group of 3 normal dogs and 19 alloxan-diabetic dogs was used in this study. The diabetes was induced by a single intravenous injection of alloxan (55 mg./Kg.). The blood glucose was determined hourly for the first 9 hours after the injection and then daily for the next 3 days. After permanent fasting hyperglycemia had been established, daily intra-arterial injections of crystalline zinc insulin (Iletin, Lilly) were given into the right femoral artery at a point proximal to the center of the femoral triangle. The injections were made under the usual aseptic precautions with a 25-gage needle. Production of hematomas was avoided by applying pressure after the injection. The diabetic dogs received from 1 to 3 units of insulin per Kg., depending on their fasting blood sugar level. The normal dogs received 1 unit of insulin per Kg.

The same portion of the right femoral artery was used for injection throughout the experiment to confine the trauma to a minimal segment of the artery. An equal volume of sterile normal saline solution was injected daily into the left femoral artery. Daily intra-arterial injections of regular insulin and normal saline solution were given for 1 to 28 weeks to the diabetic dogs and for 33 weeks to the normal dogs.

A constant diet consisting of a mixture of a low-fat commercial dog food (Kibbies) with lard and horsemeat was fed throughout the experiment. The mixture contained 18 per cent fat, 40 per cent carbohydrate and 15 per cent protein. The fat provided approximately 42 per cent of the total calories.

The diabetic dogs were sacrificed and autopsied at intervals from 1 to 28 weeks and samples of artery and muscle tissues were taken from both hind legs at constant specified levels. These tissue

From the Jay Phillips Research Laboratory, Mount Sinai Hospital, the Departments of Medicine, Physiological Hygiene and Surgery, and the Graduate School of the University of Minnesota, Minneapolis, Minn.

Supported by grants of the John A. Hartford Foundation and the Mount Sinai Hospital Research Fund.

The results reported are a part of the thesis submitted by Anatolio B. Cruz, Jr., in partial fulfillment of the requirements for the Master's Degree in Surgery.

Received for publication July 7, 1960.

Table 1
*Blood Sugar, Serum Total Cholesterol and Serum Esterified Fatty Acids in Dogs,
 Before and After the Development of Alloxan Diabetes*

Dog No.	Before injection of alloxan			After development of diabetes		
	Blood sugar mg./100 ml.	Serum total cholesterol mg./100 ml.	Serum esterified fatty acids mEq./L.	Blood sugar mg./100 ml.	Serum total cholesterol mg./100 ml.	Serum esterified fatty acids mEq./L.
1723	63	150	9.3	344	373	18.6
1727	62	117	8.9	249	415	20.1
1733	63	87	8.3	325	285	15.9
1773	77	173	12.1	240	421	23.1
1793	59	165	11.4	314	406	19.7
1785	63	116	10.0	225	306	17.2
1728	58	87	7.3	228	316	16.6
1578	51	145	12.0	331	321	19.3
Means	62	130	9.9	282	355	18.8
SEM	±2.6	±11.8	±0.63	±18.0	±19.3	±0.81

samples were analyzed for cholesterol and total fatty acid content. Microscopic studies* of the tissues were carried out using hematoxylin-eosin stains and special sudan stains for fats. Throughout the study, weekly determinations were done of fasting blood glucose,¹¹ serum cholesterol¹² and serum esterified fatty acid¹³ levels.

The following methods were used in the estimation of tissue cholesterol and total fatty acid content. Samples of tissue weighing 50 to 150 mg. were placed in containers and dried to constant weight in vacuo at 70 C. and 40 mm. of Hg pressure. Thirty per cent potassium hydroxide (0.2 ml.) was added to each sample and heated in a water bath at 90 C. until all the tissue was in solution. Then 5 ml. of water and 5 ml. of 95 per cent ethyl alcohol were added, mixed, and the solution extracted twice with 10-ml. portions of petroleum ether. After the evaporation of the petroleum ether extract, 5 ml. of ethyl alcohol-acetone (1:1) was added to dissolve the residue and the cholesterol was precipitated with digitonin. The cholesterol was then determined by the method of Abell et al.¹² The aqueous phase of the above sample was acidified with 1.0 ml. of 25 per cent hydrochloric acid and extracted with 10 ml. of petroleum ether. Aliquots of the petroleum ether phase were pipetted into weighed beakers and the solvent evaporated. The dried beakers were weighed to constant weight to determine gravimetrically the total tissue fatty acids.

*These studies were done by the courtesy of Dr. D. Gleason, Chief of Pathology, Veterans Administration Hospital, Minneapolis, Minn.

Results

The injection of alloxan produced a sustained hyperglycemia. Fasting hyperglycemia was observed in all the dogs 36 hours after the alloxan injection. There were differences among individual animals in the severity of the diabetes mellitus which persisted throughout the period of study. Along with the sustained hyperglycemia there was an increase of the total serum cholesterol and of the total esterified fatty acids. Table 1 gives the serum values before and after intravenous injection of alloxan for 8 of the dogs. No correlation between the fasting blood-sugar level and the serum-cholesterol level was found in the alloxan-diabetic dogs.

After the intra-arterial insulin, no hypoglycemic manifestations were observed at any time. In 2 dogs, a slight decrease of the venous blood sugar level was found 1 hour after the injection (16 and 25 mg./100 ml., respectively).

The chemical analysis of the arteries and muscles showed an increase of cholesterol and total fatty acids in both tissues of the insulin-injected side as compared with the saline-injected side. The data are summarized in table 2.

The mean arterial tissue cholesterol of the 19 dogs was 487 ± 32.4 mg./100 Gm. of dry tissue for the insulin-injected arteries of the

Table 2

Effect of Intra-arterial Insulin Injection (Right Femoral Artery) on the Cholesterol and Total Fatty Acids Content of the Artery and Muscle*

Dogs	No.	Artery				Muscle			
		Cholesterol		Total fatty acids		Cholesterol		Total fatty acids	
		Right	Left	Right	Left	Right	Left	Right	Left
Diabetic	19	487 ±32.4	401 ±37.3	9.88 ±1.40	2.26 ±0.33	370 ±35.4	342 ±40.5	13.14 ±1.21	6.34 ±0.82
Difference right minus left		86		7.62		28		6.80	
P		<0.012		<0.0001		>0.4		<0.0001	
Normals	3	359 ±10.6	313 ±27.2	4.1 ±1.00	2.7 ±0.71	172 ±25.6	179 ±11	6.1 ±0.84	9.0 ±3.12

*Figures are means and standard errors of the mean.
Cholesterol, mg./100 Gm. dry tissue.
Total fatty acids, Gm./100 Gm. dry tissue.

right legs, as compared with 401 ± 37.3 mg. for the saline-injected arteries of the left legs. This difference of arterial tissue cholesterol between the 2 leg arteries was significant ($P < 0.012$; see table 2). The mean artery tissue total fatty acid of the right leg in the 19 dogs receiving daily intra-arterial insulin was found to be 9.88 ± 1.40 Gm. per cent as compared to 2.26 ± 0.33 Gm./100 in the daily intra-arterial saline-injected legs of the left side. The difference of total fatty acid content of arterial tissues was found to be significant ($P < 0.0001$). The differences in lipid content were noticeable in all the animals injected 4 weeks or more, but no relationship was observed between the magnitude of the difference and the total duration of the treatment.

Similar analyses were done on the muscles of the legs of the alloxan-diabetes-mellitus dogs. The mean muscle cholesterol of 19 dogs on the daily intra-arterial-insulin-injected right leg was found to be 370 ± 35.4 mg. per cent. The difference of muscle cholesterol between the 2 legs was not significant ($P > 0.4$). However, the mean muscle fatty acid content of the daily intra-arterial-insulin-injected right leg was found to be 13.14 ± 1.21 Gm. per cent compared to the intra-arterial-saline-injected left leg of 6.34 ± 0.82 Gm. per cent. The difference of muscle fatty acid content between the right (insulin) and the left

(saline) leg was found to be significant ($P < 0.0001$).

In the 3 normal dogs the differences of cholesterol and of fatty acids content between the right and left artery were smaller than those observed in the diabetic animals and did not reach statistical significance ($P = 0.33$ for cholesterol; $P = 0.08$ for fatty acids). No differences were noted in the muscle.

Microscopic studies of the arteries and muscles were done on both legs. After daily intra-arterial insulin administration, the right legs showed proliferative changes and thickening of the media of the arterial wall; this was also noted in the arterioles of the right leg. These changes were found only in the dogs that received daily intra-arterial insulin for 26 to 28 weeks. The left leg given intra-arterial saline showed no microscopic changes. Special fat stains of the above tissue were unrevealing.

Discussion

There are many studies which indicate that insulin influences the metabolism of fat. It has been found that one of the main defects in diabetic rats is the inability of the animal to synthesize fat.¹⁴ Similarly, in vitro studies have shown that liver slices from alloxan-diabetic rats have greatly diminished ability to synthesize long chain fatty acids from C¹⁴-labeled glucose.^{15, 16}

In normal man, it was recently demonstrated that intra-arterial administration of

insulin caused a significant decrease of the arterial concentration and the arteriovenous difference of the nonesterified fatty acids.¹⁷ This result is interpreted as indication of a local effect of the insulin on the tissues of the injected leg, causing a decrease in the release of nonesterified fatty acids to the venous blood.

In the present study, intra-arterial injection of insulin was found to increase the lipid content of the artery and muscle in alloxan-diabetic dogs. This result is compatible with the idea that the insulin has a local effect on the tissues of the arterial wall and the muscle of the injected leg. Combination of insulin with the tissue as a first step of insulin action has been demonstrated by Stadie and co-workers¹⁸⁻²⁰ for muscle, tissue of the mammary gland and adipose tissue. The increased lipid content of the arterial wall observed in the present experiments can be explained then as the consequence of a stimulation of the deposition or of the synthesis (or both) of lipids produced by the insulin combined with the arterial tissue.

Our normal dogs seem to respond to the intra-arterial injection of insulin with differences of lipid content between the injected and the noninjected side which are smaller than those observed in the diabetic animals. The number of dogs in the control group is too small to warrant definite conclusions, but the result indicates a quantitative difference between the diabetic and the normal dogs with respect to the response of the arterial tissue to the intra-arterial injection of insulin. This difference may be related to the higher concentration of glucose and lipids in the blood of the diabetic animal.

It is interesting to note that both the artery and the muscle of the diabetic animals have a higher cholesterol content than those of the normal animals.

An exaggeration of the arteriosclerotic process in diabetic animals treated with insulin has been observed in animal studies. The feeding of high-cholesterol diets to alloxan-diabetic rabbits causes only a slight degree of atherosclerosis. However, when insulin is given to control the diabetes and while

the animals are on the high-cholesterol diet, marked atherosclerosis develops in all the animals.¹⁰ Studies of a similar nature were done with depancreatized chicken which developed atherosclerosis on a high-cholesterol diet. The atherosclerotic lesions regressed when the animals were transferred to a nonatherogenic diet, but the regression was prevented when insulin was given in addition.⁷

The histological studies have shown intimal and medial proliferation at different levels of the insulin-injected artery. No such changes were observed in the artery injected with saline. Whether insulin administration is a significant factor in the development of vascular degeneration in humans suffering from diabetes mellitus and treated with insulin is, of course, not settled by this study. Our results, however, clearly indicate that insulin injected into the artery of diabetic dogs increases the content of fatty acids and of cholesterol in the arterial wall.

Summary

Insulin and saline were injected into the right and left femoral arteries respectively of 19 alloxan-diabetes-mellitus dogs for 1 to 28 weeks. A significant increase of artery tissue cholesterol and total fatty acids was found on comparing the insulin-administered right leg with the saline-administered left leg. Similarly, a significant increase of total fatty acids in muscle was found on comparing the right with the left leg of the alloxan-diabetes-mellitus dogs. No significant differences were observed in the normal animals when the insulin-injected side was compared with the noninjected.

Acknowledgment

The authors are grateful for the untiring efforts, technical help and skill of Miss Donna E. Swenson, B.S., and for the invaluable assistance of Mrs. Ardeth Kapsner-Fischer.

References

1. SKOUBY, A. P.: Vascular lesion in diabetes with special references to influence of treatment. *Acta med. scandinav.* 155 (suppl. 317): 1, 1956.
2. VAN ECK, W. F.: Effects of a low fat diet on the serum lipids in diabetes and its significance in diabetic retinopathy. *Am. J. Med.* 27: 196, 1959.

3. BRYFOGLE, T. W., AND BRADLEY, R.: Vascular complications of diabetes mellitus. *Diabetes* 6: 159, 1957.
4. BELL, A. T.: Postmortem study of vascular disease in diabetics. *A.M.A. Arch. Path.* 53: 444, 1952.
5. DOLGER, H.: Clinical evaluation of vascular damage in diabetes mellitus. *J.A.M.A.* 134: 1289, 1947.
6. DOLGER, H.: Factors influencing premature cardiovascular degeneration in diabetes mellitus. *Progress Clin. Endocrinol.*, edited by S. Soskin. New York, Grune & Stratton, 1950, pp. 303-306.
7. PICK, R., STAMLER, J., AND KATZ, L. N.: Influence of hormones on lipids and atherosclerosis in experimental animals. *In Hormones and Atherosclerosis*, edited by G. Pincus. New York, Academic Press Inc., 1959, pp. 173-193.
8. DUFF, G. L., AND McMILLAN, G. C.: Effect of alloxan diabetes on experimental cholesterol atherosclerosis in the rabbit. *J. Exper. Med.*, 89: 611, 1949.
9. MCGILL, M. G., AND HOLMAN, R. L.: Influence of alloxan diabetes on cholesterol atheromatosis in the rabbit. *Proc. Soc. Exper. Biol. & Med.* 72: 72, 1949.
10. DUFF, G. L., BRECHIN, D. J. A., AND FINKELSTEIN, W. E.: Effect of alloxan diabetes and experimental atherosclerosis in the rabbit. *J. Exper. Med.* 100: 371, 1954.
11. SOMOGYI, M.: New reagent for the determination of sugar. *J. Biol. Chem.* 160: 61, 1945.
12. ABELL, L. L., LEVY, B. B., BRADIE, B., AND KINDELL, F. E.: Simplified method for the estimation of total cholesterol in serum and demonstration of its specificity. *J. Biol. Chem.* 195: 357, 1952.
13. BAUER, F. C., AND HIRSCH, E. F.: New method for the colorimetric determination of the total esterified fatty acids in human sera. *Arch. Biochem.* 20: 242, 1949.
14. STETTEN, DE W., JR., AND BOXER, G. E.: Studies in carbohydrate metabolism: Metabolic defects in alloxan diabetes. *J. Biol. Chem.* 156: 271, 1944.
15. BRADY, R. O., AND GURIN, S.: Biosynthesis of fatty acids and cholesterol in experimental diabetes. *J. Biol. Chem.* 187: 589, 1950.
16. CHERNIK, S. S., CHAIKOFF, I. L., MASORO, E. J., AND ISAEFF, E.: Lipogenesis and glucose oxydation in liver of alloxan diabetic rat. *J. Biol. Chem.* 186: 527, 1960.
17. ESTES, E. H., BOGDONOFF, M. D., FRIEDBERG, S. J., HARLAN, W. R., AND TROUT, D. L.: Effect of insulin on nonesterified fatty acid release from the human leg. *J. Clin. Invest.* 38: 2131, 1959.
18. STADIE, W. C., HAUGAARD, N., MARSH, J. B., AND HILLS, A. G.: Chemical combination of insulin with muscle (diaphragm) of normal rat. *Am. J. Med. Sc.* 218: 265, 1949.
19. HILLS, A. G., AND STADIE, W. C.: Effect of combined insulin upon the metabolism of the lactating mammary gland of the rat. *J. Biol. Chem.* 194: 25, 1952.
20. HAUGAARD, N., AND MARSH, J. B.: Effect of insulin on the metabolism of adipose tissue from normal rats. *J. Biol. Chem.* 194: 33, 1952.

Circulation Research

JOURNAL OF THE AMERICAN HEART ASSOCIATION



Effect of Intra-arterial Insulin on Tissue Cholesterol and Fatty Acids in Alloxan-Diabetic Dogs

ANATOLIO B. CRUZ, Jr., DONALD S. AMATUZIO, FRANCISCO GRANDE and LYLE J. HAY

With the technical assistance of Miss Donna E. Swenson, B.S.

Circ Res. 1961;9:39-43

doi: 10.1161/01.RES.9.1.39

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 1961 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circres.ahajournals.org/content/9/1/39>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation Research* is online at:
<http://circres.ahajournals.org/subscriptions/>