The Effect of Insulin on the Incorporation of Sodium (1-¹⁴C)-Acetate into the Lipids of the Rat Aorta*

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Summary. Experiments were performed to test the hypothesis that insulin has a role in the pathogenesis of atherosclerosis. Rats were injected intravenously with sodium (1-¹⁴C)-acetate (10 μ Ci per 100 g weight) with and without insulin (100 000 μ units per 100 g weight). After one hour, they were killed, and the lipids extracted from the cleaned aortas. The lipids were separated by thin layer chromatography, and the radioactivity in the total lipids and the lipid fractions was measured. The results showed that insulin stimulated the incorporation of sodium (1-¹⁴C)-acetate into total lipids, cholesterol and phospholipids to a significant extent. Incorporation into triglyceride just failed to reach statistical significance. These results are compatible with some of the features of human atherosclerosis, and support the hypothesis that insulin and atheroma are causally linked. The finding that insulin had an effect on cholesterol metabolism was unexpected and warrants further investigation.

Effet de l'insuline sur l'incorporation de l'acétate de sodium (1-¹⁴C) dans les lipides de l'aorte chez le rat

Résumé. On a soumis à l'expérience l'hypothèse que l'insuline joue un rôle dans la pathogénèse de l'athérosclérose. De l'acétate de sodium 1-¹⁴C (10 μ Ci par 100 g de poids) avec et sans insuline (100 000 μ unités par 100 g de poids) a été injecté à des rats, par voie intra-veineuse. Une heure après, ils ont été tués et les lipides extraits de l'aorte nettoyée. Les lipides ont été séparés par chromatographie en couche mince et la radioactivité a été mesurée dans les lipides totaux et les fractions de lipides. Les résultats ont montré que l'insuline a stimulé l'incorporation de l'acétate de sodium 1-¹⁴C dans les lipides totaux, le cholestérol et les phospholipides d'une façon significative. L'incorporation dans les triglycérides n'atteint

pas la significativité. Ces résultats sont compatibles avec quelques aspects de l'athéro-sclérose chez l'homme et viennent appuyer l'hypothèse d'une étroite liaison entre l'insuline et l'athérome. On ne s'attendait pas à découvrir que l'insuline avait un effet sur le métabolisme du cholestérol et cela implique de nouvelles recherches.

Die Einwirkung von Insulin auf die Inkorporation von (1-¹⁴C)-Natriumacetat in die Lipide der Rattenaorta

Zusammenfassung. Es wurden Versuche durchgeführt, um die Hypothese zu prüfen, ob Insulin eine Rolle in der Pathogenese der Arteriosklerose spielt. Zu diesem Zweck wurden Ratten intravenös (1-¹⁴C-Natriumacetat (10 μ Ci pro 100 g Körpergewicht) mit und ohne Insulin (100 000 μ E pro g Gewicht) injiziert. Nach einer Stunde wurden die Tiere getötet und die Lipide aus den gereinigten Aorten extrahiert. Die Lipide wurden durch Dünnschichtchromatographie getrennt, dann wurde die Radioaktivität in den Gesamtlipiden und den Lipidfraktionen gemessen. Die Ergebnisse zeigten, daß Insulin die Inkorporation von (1-¹⁴C)-Natriumacetat in die Gesamtlipide, das Cholesterin und die Phospholipide in signifikantem Ausmaße stimuliert. Die Inkorporation in die Triglyceride war gerade eben statistisch nicht signifikant. Diese Ergebnisse sind vereinbar mit einigen Charakteristika der menschlichen Arteriosklerose und unterstützen die Hypothese, daß Insulin und Atherom ursächlich verknüpft sind. Der Befund, daß Insulin eine Einwirkung auf den Cholesterinstoffwechsel hat, war unerwartet und erfordert weitere Untersuchungen.

Key-words: Atherosclerosis, cholesterol, phospholipids, insulin, aorta.

Introduction

The majority of patients with ischaemic vascular disease have elevated insulin responses to glucose, with or without high fasting serum insulin levels [14, 15, 3, 11, 28]. Hyperinsulinism is present long after the acute episode of myocardial infarction, and is also found in subjects with angiographically proven coronary artery disease without occlusion. This suggests that hyperinsulinism is related to the underlying disease process, rather than being a metabolic response to an acute illness. In studies in which several metabolic abnormalities have been investigated, hyperinsulinism has

been the commonest abnormality in ischaemic vascular disease [28].

In addition, many of the other features associated with ischaemic vascular disease, including obesity, hyperlipidaemia, hyperglycaemia, increased dietary carbohydrate, and lack of physical exercise, are themselves associated with elevated serum insulin levels [24]. A hypothesis has been presented suggesting that hyperinsulinism is the primary abnormality in atherosclerosis [24] and insulin stimulation of lipid synthesis in arterial tissue has been reported [22, 23].

This paper describes some detailed studies on the effect of insulin on the lipid metabolism of the arterial wall.

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Methods

The method used was an adaptation of procedures designed to study the action of insulin on muscle and adipose tissue *in vivo* [16]. A description of the materials will first be given, followed by an account of the procedure.

to make a concentration of 40000 μ units per ml, i.e. each animal received 100000 μ units per 100 g body weight. The control and insulin-containing solutions were of the same volume and contained the same quantity of labelled sodium acetate.

Thin layer chromatography was carried out on Silica Gel G (250 μ thick). Each plate was divided into

Fig. 1. Section of rat aorta ($\times 200$) showing absence of fatty adventitious tissue

Materials

The animals were male rats of the Wistar strain, and weighed 90–120 g, all the animals in one experiment being the same weight. They were fed commercial rat chow and not starved prior to the experiments. No anaesthetic was necessary. Six to ten rats were used in each experiment and were divided into two equal groups, one group receiving the insulin-containing solution, the other an otherwise identical solution containing no insulin.

The injection solution consisted of Gey and Gey buffer, radio-labelled sodium acetate, and, for half the animals, insulin. The Gey and Gey buffer [9] was prepared weekly from stock solutions. The sodium ($1\text{-}^{14}\text{C}$)-acetate (The Radiochemical Centre, Amersham, England; specific activity 40.0 mCi/mM) was dissolved in buffer to make a final concentration in the injection solution of 4 $\mu\text{Ci/ml}$, i.e. each animal received 10 μCi per 100 g body weight. The insulin was five times recrystallized insulin (Boots Pure Drug Company, Nottingham, England; potency 22.2 units per mg) initially dissolved in 0.6% acetic acid. The solution, which was freshly prepared each week, was stored at 5°C. Insulin was added to the buffer solution

six vertical strips, each 3 cm wide, one strip being further divided into three, for the standard solutions. The plates were developed in a vertical tank containing 100 ml of hexane: diethyl ether: acetic acid (90:15:1). The lipids were stained with iodine vapour.

Liquid scintillation counting was performed in a Packard Tri-Carb Scintillation Spectrometer. The scintillation fluid contained 4 g of 2,5-diphenyloxazole and 40 ml of 1,4-di-2-(5-phenyloxazolyl) benzene in 1 l of toluene.

Procedure

The rats, which were numbered and weighed, were injected in turn with 2.5 ml per 100 g body weight of the injection solution. The injection was made into the tail vein which had been dilated by warming.

One hour after injection the animals were killed by a blow on the head. On some occasions they were then immediately decapitated and blood collected from the neck vessels. The aortas were dissected out and carefully cleaned of adventitious tissue with the aid of a dissecting microscope. Histological examination of the aortas confirmed that they were free of fatty material

(Figure). The cleaned aortas were refluxed in 1 ml chloroform-methanol (2:1) for three hours to extract the lipids. The aortas were then washed, allowed to dry in air, and weighed.

The chloroform-methanol solution was washed by the method of Folch *et al.* [8]. This procedure removes more than 99% of water-soluble impurities including

development and staining, the lipids were identified and marked, and the stain allowed to fade. The areas of silica gel containing the lipids were scraped off the plates into counting vials, and the lipid eluted with 5 ml scintillation fluid [26]. The intervening areas of silica gel were also tested and showed no radioactivity.

A quench correction curve for differing amounts of

Table 1

Substrate — Sodium acetate-1-¹⁴C
Insulin — 100 000 μ U/100 g body wt.
Time — 1 h

Aortic lipid radioactivity
(Mean CPM per mg dry wt. $\pm$ SEM)

	<i>Control</i>	<i>Test</i>	<i>t</i>	<i>P</i>
Total lipid	33.35 $\pm$ 1.50 (38)	46.50 $\pm$ 2.50 (37)	4.53	<0.001
Cholesterol	1.44 $\pm$ 0.12 (38)	1.77 $\pm$ 0.15 (34)	1.78	<0.05
Fatty acids	1.11 $\pm$ 0.84 (42)	1.16 $\pm$ 0.76 (36)	0.04	0.5
Triglyceride	7.33 $\pm$ 0.84 (42)	9.16 $\pm$ 1.13 (36)	1.31	0.10 > <i>P</i> > 0.05
Phospholipids	13.37 $\pm$ 1.23 (39)	20.58 $\pm$ 1.86 (36)	3.21	<0.01

(Numbers in parentheses refer to number of observations)

Table 2

Substrate — Sodium acetate-1-¹⁴C
Insulin — 100 000 μ U/100 g body wt.
Time — 1 h

Serum lipid radioactivity
(Mean CPM per ml $\pm$ SEM)

	<i>Control</i>	<i>Test</i>	<i>t</i>	<i>P</i>
Cholesterol	101.14 $\pm$ 12.60 (13)	136.42 $\pm$ 33.0 (13)	0.99	>0.20
Fatty acid	117.52 $\pm$ 13.71 (13)	174.32 $\pm$ 38.42 (13)	1.52	0.10 > <i>P</i> > 0.05
Triglyceride	463.12 $\pm$ 80.19 (13)	933.16 $\pm$ 302.05 (13)	1.50	0.10 > <i>P</i> > 0.05
Phospholipids	214.45 $\pm$ 31.47 (13)	280.72 $\pm$ 55.35 (13)	1.07	<0.20

(Numbers in parentheses refer to number of observations)

Table 3

Correlation coefficients
Serum and aortic lipids
Substrate — Sodium acetate-1-¹⁴C
Insulin — 100 000 μ U/100 g body wt.

	<i>Control</i>			<i>Test</i>		
	<i>r</i>	<i>t</i>	<i>p</i>	<i>r</i>	<i>t</i>	<i>p</i>
Cholesterol	-0.04	0.12	>0.49	-0.12	0.38	>0.35
Free fatty acids	-0.09	0.28	>0.35	-0.40	1.33	>0.10
Triglyceride	0.06	0.19	>0.40	-0.17	0.54	0.30
Phospholipid	-0.41	1.39	0.10	0.13	0.41	>0.30

sodium acetate. Two samples of 0.5 ml were evaporated to dryness in counting vials, and the radioactive lipid redissolved in 5 ml scintillation fluid for estimation of total lipid radioactivity. The remaining solution was evaporated to small volume preparatory to chromatography.

The lipid solution was applied to the origin of the thin layer plate in a thin line. Samples from control and insulin-treated animals were run on alternate strips, and standard solutions were run on each plate. After

silica gel was constructed and showed no effect of this substance on counting efficiency which was 80% throughout. After subtraction of background counts and correcting for efficiency, the results were expressed as counts per minute per milligram dry aortic weight.

Serum lipids were extracted in chloroform-methanol [10]. Purification, separation and counting procedures were identical to those for aortic lipids. The results were expressed as counts per minute per millilitre of serum.

Results

The results are expressed in terms of dry defatted aortic weight. It has been shown that in arterial tissue the ratio of DNA to weight is constant at all ages, so that the aortic weight reflects the cell content of the tissue [5].

The radioactivity in the aortic lipids in the control and treated animals is shown in Table 1. The significance of the differences were tested by the Student 't' test, and the probability values are shown in the table. It can be seen that the radioactivity in the total lipid, cholesterol and phospholipid fractions is significantly higher in the insulin-treated (test) group, and the triglyceride value is also higher although the difference does not quite reach statistical significance. The cholesterol result has already been the subject of a preliminary report [23], and the results here, which come from a much larger number of aortas, confirm the early report.

The radioactivity in the serum lipids, taken simultaneously with the aortic lipids are shown in Table 2. Serum lipids were measured in only a proportion of animals studied. The results are quite different from those in the aortic lipids. The difference in any group does not reach significance at the 5% level, although those in the fatty acid and triglyceride fractions just fail to do so. It is notable that insulin has no significant effect on serum cholesterol and phospholipid radioactivity.

Correlation coefficients between corresponding aortic and serum lipid radioactivities were calculated using the r to z transformation for small numbers [7]. The results, shown in Table 3, show no significant correlation between serum and aortic lipids.

Discussion

The results of the experiments reported here show that the incorporation of acetate into the cholesterol, phospholipids and triglyceride of the arterial wall can be enhanced by insulin. The exact site of action of the insulin cannot be determined in an experiment such as this. The studies on serum lipid radioactivity and the lack of correlation between serum and aortic lipid values suggest that insulin exerts its effect on the arterial wall itself, rather than by way of serum lipid abnormalities. However, the measurements were made one hour after the injection of insulin and isotope, and an earlier effect on serum lipids with infiltration of these into the arterial wall cannot be entirely excluded. In addition, the number of analyses of serum lipid radioactivity was smaller than those of the aortas, and an insulin effect on serum lipids may have been missed. Subsequent experiments, using technique *in vitro* have however confirmed that insulin has a direct stimulating action on the lipid metabolism of the arterial wall [20].

The fact that an insulin effect on aortic lipid metabolism was found so soon after injection suggests that stimulation of lipogenesis rather than inhibition of lipolysis is the mechanism involved. Moreover, the effect is not transitory, as it has been shown that long-term administration of insulin results in the accumulation of histologically stainable lipid in the arterial wall [21].

The marked effect of insulin on arterial metabolism *in vivo* is in contrast to the lack of any such effect *in vitro* [29]. This is despite the fact that the aortas in the studies *in vitro* were exposed to far higher concentrations of both isotopes and insulin. The reason for the discrepancy is unknown, although similar situations have been reported. Thus insulin *in vitro* has no effect on the impaired metabolism of aortas from alloxanized animals, but prior treatment of the animals with insulin restores to normal the metabolism of the aortas *in vitro* [12]. The situation *in vitro* may be deficient in the absence of intraluminal pressure, the absence of adventitious tissue from the aortas, damage to the aortas during preparation, or some deficiency in the incubation medium. Which of these is true is unknown.

The relationship of the metabolic studies reported here to human atherosclerosis must be assessed. The first problem is whether the results of work on animal tissues can be applied to humans. Structurally, the rat aorta resembles the human aorta, being predominantly composed of fibrous and elastic tissue. The rat is well known to be resistant to experimental atherosclerosis [25]. However, spontaneous vascular lesions do occur in old rats, and these are qualitatively similar to those seen in humans [25]. As human atherosclerosis takes several decades to develop, it could be said that the human is also resistant to atherosclerosis and should therefore be compared with animals which do not develop lesions easily.

There have been many descriptions of the chemical composition of human atheromatous lesions. These analyses have been performed on established lesions and therefore cannot be directly compared with metabolic studies, which must represent the very earliest abnormalities. The reports however agree that the advancing atheroma is characterized by increasing deposition of total lipids, cholesterol and phospholipids in the arterial wall [2, 1, 27]. There is controversy over the importance of triglyceride in the atheromatous plaque, but several authors have shown that it increases in amount with progressive disease [2, 1]. The lipids of the human atheromatous lesion are therefore similar to those that have been studied in the rat aorta.

Stimulation of triglyceride synthesis in the arterial wall by insulin would be similar to its action on adipose tissue metabolism [18]. However, insulin is not generally considered to influence phospholipid or cholesterol synthesis. Insulin strongly stimulates the synthesis of fatty acids in adipose tissue [31] and the same process may occur in arterial tissue, although the results shown here do not confirm this. Phospholipids are formed

from fatty acids [17], and this may explain the increase in radioactive phospholipids in the insulin-treated aortas and the apparent lack of effect on fatty acids.

There are a few published reports suggesting that insulin has some effect on cholesterol metabolism. Insulin inhibits the regression of atheromatous lesions which occurs when cholesterol-fed chicks are transferred to a normal diet [19], and causes increased deposition of cholesterol when infused into the arteries of cholesterol-fed dogs with alloxan diabetes [4]. In pancreatectomized rats fed an atherogenic diet, treatment with insulin reduces the serum cholesterol to that of non-diabetic controls, but does not decrease the incidence or severity of their vascular lesions, as happened when a comparable reduction of hypercholesterolaemia was effected by dietary means [30]. In dogs, the elevation of serum insulin levels to over double their normal value by diverting pancreatic venous blood into the inferior vena cava and feeding sucrose results in a two-fold increase in serum cholesterol levels [6]. There is thus experimental evidence for an effect of insulin on cholesterol metabolism.

The exact site of the action of insulin on cholesterol metabolism is not clear. Cholesterol is synthesized from acetate by a long and complicated metabolic pathway involving at least thirty enzymic reactions. The rate limiting step is the conversion of B-hydroxy-B-methyl-glutaryl-coenzyme A to mevalonic acid [13].

Two possible explanations for the effect of insulin on cholesterol synthesis are:

- a) acetate passes into the cells with glucose, a step which is accelerated in the presence of insulin; the increase in cholesterol synthesis would then simply be due to increased availability of labelled acetate in the aortas of the insulin-treated animals;
- b) the increased glucose metabolism in the insulin-treated animals may stimulate rates of lipogenesis associated with increments in the activities of enzymes in the hexose monophosphate oxidative pathway; thus cholesterol metabolism would be accelerated.

Whatever the mechanism, this was an unexpected finding and one which warrants further investigation. It would be interesting to know whether the effect of insulin on arterial cholesterol is unique to this tissue, or whether the major sites of cholesterol metabolism, the liver and the gastro-intestinal tract, are similarly affected.

Epidemiological evidence has shown that hyperinsulinism is the common factor in many of the metabolic abnormalities associated with atherosclerotic vascular disease [24]. The experiments described here show that treatment with insulin results in increased deposition of lipids in the arterial wall of experimental animals. The speculation is therefore justified that insulin may have a causative role in the pathogenesis of atherosclerosis.

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