Lipids in the Pulmonary Artery and the Lungs of Severely Diabetic Rats

A Histochemical and Chemical Study

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Summary. To investigate the effect of diabetes on the lipid composition of the lungs and of the pulmonary artery, 43 streptozotocin diabetic rats and 43 control rats were examined. Triglyceride deposits were observed by a histochemical method in the branches of the pulmonary artery in 10 diabetic rats but in none of the controls. In the pulmonary tissue of the diabetic rats the total lipid content was not different from that of control animals, but the relative amount of phospholipids was decreased ($p < 0.001$), and that of non-esterified fatty acids ($p < 0.001$) and triglycerides ($p < 0.05$) increased as compared to the control rats. These results indicate abnormalities in the lipid metabolism of the pulmonary artery and lungs during insulin deficiency.

Key words: Diabetes, non-esterified fatty acids, lipid metabolism, lung, phospholipids, pulmonary artery, rat, streptozotocin, triglycerides.

The lipid composition and metabolism of the lungs in diabetes has been little studied. According to Uspenskii [1], lipolytic activity is increased in the lungs of diabetic rats. A marked increase in pulmonary phospholipids and a decrease in pulmonary triglycerides in alloxan-diabetic rabbits has been noted by Stratta Monga et al. [2]. Cardellino and co-workers [3] assumed that defective synthesis of lecithin may account for part of the lipid changes of the lung in diabetes.

In autopsy studies one of us has recently observed perivascular foam cell deposits in the lungs of diabetic subjects [4]. Therefore we decided to investigate the lipid composition of the lungs and pulmonary artery in experimental diabetes.

Material and Methods

Animals

Male Sprague-Dawley rats weighing from 300 to 350 g were used. They were fed ad libitum a normal commercial chow containing 53.0% carbohydrate, 20.9% protein, 4.5% fat, 3.9% fibre, and the usual vitamins and minerals. The total series consisted of 43 diabetic and 43 control rats.

The Induction of Diabetes

Diabetes was induced with intracardially administered 3% solution of streptozotocin (Upjohn & Co., lot U-9889 and lot 60, 140-MCM) which was freshly prepared in 0.1 molar citrate buffer, pH 4.5, and injected in a dose of 65 mg/kg of body weight into animals under pentobarbital anaesthesia [5]. The control rats received an equal volume of buffer intracardially. Only those animals which were severely diabetic (weight loss, marked ketonuria, Ketotix® + + +, and glucosuria 5%) were used for the study. The rats were not treated with insulin and were killed four days after the injection of streptozotocin.

Treatment of Blood and Tissue Specimens

Blood was drawn from the retro-orbital venous plexus of non-fasting rats through a capillary tube during pentobarbital anaesthesia and collected in ice-cold EDTA tubes (final concentration of EDTA 1 mg/ml). The plasma specimens were stored at -20°C until analysis. Immediately after blood sampling, the animals were killed by decapitation. The lungs were removed and one lung was perfused through the pulmonary artery with 0.9% saline in

Table 1. The non-fasting levels of blood glucose, plasma triglycerides, non-esterified fatty acids, phospholipids and total cholesterol in diabetic rats four days after the injection of streptozotocin (65 mg/kg) and in control rats (Mean±SEM)

	Diabetic rats n = 43	Control rats n = 43	p
Blood glucose (mg/100 ml)	312±5.0	101±2.1	<0.001
Plasma triglycerides (mg/100 ml)	987±100	182±9.8	<0.001
Plasma non-esterified fatty acids (mmol/l)	1.16±0.08	0.51±0.04	<0.001
Plasma phospholipids (mg/100 ml)	251±18.3	118±4.1	<0.001
Plasma total cholesterol (mg/100 ml)	122±8.2	72±4.6	<0.001

Table 2. The content of non-esterified fatty acids, triglycerides, total cholesterol and phospholipids of the pulmonary tissue of diabetic and control rats, calculated as mg/g of freeze-dried weight and mg/g of total lipids (Mean±SEM).

Lipids in the pulmonary tissue	Diabetic rats n = 43	Control rats n = 43	p
<i>mg/g of freeze-dried weight</i>			
Non-esterified fatty acids	31±1.7	21±1.1	<0.001
Triglycerides	28±1.1	25±2.3	N. S.
Total cholesterol	20±0.8	22±0.8	N. S.
Phospholipids	99±4.1	109±3.7	0.05<p<0.10
Total lipids	170±4.4	171±4.6	N. S.
<i>mg/g of total lipids</i>			
Non-esterified fatty acids	159±6.6	118±4.4	<0.001
Triglycerides	187±8.5	155±12.0	<0.05
Total cholesterol	118±4.3	111±4.9	N. S.
Phospholipids	536±9.8	621±11.0	<0.001

order to remove the remaining blood. The perfusion was carried out with a manometer, using a constant pressure, and it was continued until the fluid coming from the pulmonary veins was clear. Thereafter a specimen of lung was set aside for histochemical analysis and another piece was separated from visible vessels for chemical analysis. A piece of pulmonary artery and of thoracic aorta was taken for histochemical analysis. In addition a sample of femoral artery was taken from 10 diabetic and 10 control rats for histochemical analysis. All tissue specimens were frozen with liquid nitrogen and stored at -70°C until analysis.

Methods

Pulmonary tissue, pulmonary artery, thoracic aorta and femoral artery were analysed histochemically for hydrophobic lipids [6], fatty acids [7], triglycerides [8], total cholesterol [9], and phospholipids [10].

After part of the lung had been freeze-dried and homogenized in chloroform-methanol (2:1), the lipids were extracted [11] and the total lipids were measured gravimetrically [12]. The dried extract was reconstituted to 1 ml with chloroform, and chemical analysis of non-esterified fatty acids (NEFA) [13], triglycerides [14], total cholesterol [15] and phospholipids [16] was performed. Blood glucose was determined according to Carrol et al. [17] with slight modifications [18], and plasma NEFA, triglycerides, total cholesterol and phospholipids were measured by methods referred to above. Values for pulmonary lipids are expressed as mg/g of total lipids and mg/g of freeze dried weight of the pulmonary tissue. Non-esterified fatty acids are expressed in terms of their equivalence in palmitic acid in mmol/l.

The results are given as mean ± SEM. The statistical significance of the differences between mean values was calculated by using Student's t-test. The difference between observed and expected frequencies was tested by the X²-test. The regression analyses were made by the method of least squares [19].

Results

During the four days after the injection of streptozotocin the diabetic rats lost 43 ± 2.1 g of weight, while the control animals gained 15 ± 2.0 g. The average daily water consumption in the diabetic animals increased from 30 to 110 ml.

Four days after the injection of streptozotocin the rats were markedly hyperglycaemic, and their plasma lipids were significantly higher than in the control animals (Table 1).

Lipids in the Arterial Wall

Hydrophobic lipids were observed in the wall of the pulmonary arteries in 10 out of 43 diabetic rats, but in none of the control animals. Further histochemical analysis proved the material to be triglycerides. The deposits were located in the media and occasionally also in the intima of pulmonary artery branches, having a diameter of 300 to 1400 µm

Fig. 1. Triglycerides in the wall of the pulmonary artery. The positive material is mostly located in tunica media, and is seen as numerous dark spots (→) (lead-lipase sulphide, original magnification $\times 400$)

(Fig. 1). The aortic wall ($n = 43$) or the femoral artery wall ($n = 10$) did not contain visible triglyceride deposits in any of the diabetic or control rats.

The mean level of plasma triglycerides was 985 ± 165 mg/100 ml in the rats having triglyceride deposits in the pulmonary artery wall ($n = 10$) and 965 ± 123 mg/100 ml in the cases with no deposits ($n = 33$). No relation was observed between the presence of pulmonary artery triglycerides and the level of plasma triglycerides. The level of plasma phospholipids was similar in these two subgroups, too (275 ± 34 and 244 ± 21 mg/100 ml, respectively).

Lipids of the Pulmonary Tissue

The content of total pulmonary lipids was identical in diabetic and control rats, when calculated as mg/g of freeze-dried weight (Table 2). The NEFA content was higher in the pulmonary tissue of diabetic than control rats, whereas the level of triglycerides and total cholesterol was similar in both groups (mg/g of freeze-dried tissue). The amount of phospholipids was somewhat lower in the diabetic than in the control rats.

When calculated as mg/g of total lipids, the increase in pulmonary non-esterified fatty acids and the decrease in phospholipids in the diabetic rats was highly significant as compared to the findings in the control rats. The level of the pulmonary triglycerides was also higher in the diabetic than in the control rats.

There was no correlation between plasma and pulmonary NEFA in the diabetic and control rats (r less than 0.1 in both groups). An inverse correlation between the amount of NEFA and phospholipids in the pulmonary tissue was observed in the diabetic rats ($r = -0.43$, $p < 0.001$), but no correlation was found in the control group. The amount of pulmonary phospholipids and triglycerides correlated inversely both in the diabetic ($r = -0.76$, $p < 0.001$) and control group ($r = -0.79$, $p < 0.001$) (Fig. 2).

The diabetic rats, which had triglyceride deposits in the pulmonary arteries, did not differ from the animals with no deposits in respect of the content of pulmonary NEFA, triglycerides, total cholesterol or phospholipids.

The histochemical analysis of the pulmonary tissue did not reveal any clear differences in the amount of lipids between the diabetic and non-diabetic rats.

Discussion

The events leading to the deposition of lipid in arterial walls are central to the pathogenesis of atherosclerosis. The majority of human and experimental arterial wall metabolism studies have been directed to the pathogenesis of fatty streaks [20]. Cholesterol derived from plasma is the lipid which accumulates in greatest quantity in the fatty streaks. The deposition of lipoprotein in the intima is also associated with an increase in phospholipid and triglyceride synthesis [20]. A significantly greater proportion of

Fig. 2. Correlation between the content of pulmonary phospholipids and triglycerides of 43 diabetic and 43 control rats

($1\text{-}^{14}\text{C}$)-oleic acid is incorporated into triglyceride and a lower proportion into phospholipid in tissue culture by the intimal cells of cholesterol-fed as compared to normal-fed rabbits [21].

The arterial smooth muscle cell contains enzymes necessary to synthesize and metabolize most if not all of the lipids, with the possible exception of cholesterol, present in the arterial wall. The predominant involvement of the intima and inner media in the development of atherosclerotic lesions suggests, however, that interactions between plasma constituents, particularly the circulating lipoproteins and the arterial wall, serve not only to modulate cellular metabolism, but that uptake of lipid or intact lipoproteins may provide an exogenous source of lipid for the artery [22]. Accordingly, one proposal of the pathogenesis of atherosclerosis, presented by Zilversmit [23], links atherogenesis to an interaction of endothelial lipoprotein lipase with circulating very low-density lipoproteins. Lipolytic activity has been observed in the arterial wall of several species. Dicorleto and Zilversmit [24] identified the lipase in bovine artery to be lipoprotein lipase, and found it to have the highest specific activity in the intimal regions of the artery. The lipase in the pig aorta, too, has been shown to be a lipoprotein lipase [25]. The amount of lipoprotein lipase activity in aortas of

different animals is variable, but the localization of the activity at the endothelial side has been consistently observed [24].

Stout [26] suggested that sustained elevation of circulating insulin plays a major role in the pathogenesis of atherosclerosis, and that arterial smooth muscle cells have thereby a key role [27]. D-glucose- $\text{U-}^{14}\text{C}$ incorporation into arterial lipids in vitro is greater in control than in streptozotocin-diabetic rats, and the correlation between this effect and plasma insulin levels is positive [28]. Stout's hypothesis is that hyperinsulinism may promote lipid deposition in the artery both by enhancing lipogenesis and inhibiting lipolysis [28].

All three forms of large vessel lesions, atherosclerosis, medial calcification and diffuse intimal fibrosis are more common in the diabetic than in the non-diabetic individual, particularly in the intermediate-sized muscular arteries [29]. The main finding of our study was the observation of considerable triglyceride deposits in the pulmonary artery wall in some of the severely diabetic rats. Although the finding occurred in only 10 out of 43 diabetic rats, we consider insulin deficiency to be the likely cause of the triglyceride deposits, because we have not found a similar change in any of 19 insulin-treated, streptozotocin-diabetic rats [30], $X^2 = 3.689$, $p < 0.05$. Two different mechanisms or a combination of them might come into question in the deposition of triglycerides in the pulmonary arteries in the severely diabetic rats: the accumulation of triglycerides could be due to hyperlipaemia [23], or to decreased activity of lipoprotein lipase, caused by insulin deficiency [31]. Even if the latter mechanism were involved, formation of triglycerides in the arterial wall might occur as the circulation would bring an abundance of substrate. No triglyceride deposits were found in the wall of the thoracic aorta or femoral artery, suggesting that the change might be specific for the pulmonary artery.

In the lungs synthesis of lipids is primarily directed toward the formation of phospholipids. The most important phospholipid in the lung is lecithin [32]. Cardellino et al. [3] showed decreased surface tension in the alveolar fluid of alloxan-diabetic rabbits and suggested the observation to indicate defective synthesis of dipalmityl-lecithin, a fundamental component of alveolar surfactant. Stratta Monga et al. [2] observed a decrease in pulmonary triglycerides and an increase in pulmonary phospholipids in alloxan-diabetic rabbits. The results are opposite to our findings. The difference might be due to alloxan, because they noticed also decreased levels of serum triglycerides, a finding typical of alloxan-diabetic animals [33]. On the contrary, in severe ex-

perimental diabetes, caused by streptozotocin, serum triglycerides are markedly elevated [34], as our results also indicate.

In the lung, as well as in other tissues, lecithin is mainly synthesized by incorporation of cytidine diphosphate choline into diglycerides [35]. Glucose is needed for the formation of glycerol, acetate and NADPH [36, 37], the last mentioned being the necessary cofactor for lipogenesis [37]. Thus the reduced levels of phospholipids in the pulmonary tissue of diabetic rats, when expressed in relation to the amount of total lipids, could be due to a low rate of phospholipid synthesis in insulin deficiency. Our results are in agreement with the findings that insulin augments phospholipid synthesis in adipose tissue *in vitro* [38] and that lack of insulin *in vitro* decreases hepatic biosynthesis of phospholipid [33].

The NEFA content is increased in the adipose tissue of diabetic rats [39]. Our results show that NEFA concentration is also higher than normal in pulmonary tissue of diabetic rats. The finding was probably not due to diffusion of NEFA from the blood, because there was no correlation between the NEFA contents in serum and pulmonary tissue.

Thus our findings suggest that pulmonary tissue probably behaves like other tissues in insulin deficiency.

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