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Accumulation of Lipid in Muscular Arteries of Short-term Diabetic Rats

An Electron Microscope Study

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Summary. Lipid accumulation in muscular (pulmonary, coronary and tibial) arteries and elastic (aorta and pulmonary) arteries of streptozotocin diabetic (65 mg/kg) rats was studied with an electron microscope. Arterial tissue specimens taken 4 days after the induction of diabetes showed lipid deposits in smooth muscle cells in the muscular arteries of 9 out of 24 diabetic rats, but in none of the 17 control rats. Histochemically the lipid was identified as triacylglycerol. Lipid accumulation was not seen in the elastic arteries of either diabetic or control rats. The diabetic animals with lipid deposits had slightly but significantly higher plasma glucose concentrations ($p < 0.02$), higher non-esterified fatty acids levels ($p < 0.01$), and lower concentrations of plasma insulin ($p < 0.02$) than those without arterial deposits. The amount of lipid deposited in the arteries was closely related to the plasma non-esterified fatty acid level, which was in the ranges 0.8–1.1 mmol/l in diabetic rats without deposits, and 1.1–2.4 mmol/l in those with deposits. The findings suggest that lipid accumulation in smooth muscle cells of muscular arteries during acute diabetes could result from the high plasma non-esterified fatty acid concentrations.

Key words: Diabetes, streptozotocin, coronary artery, muscular pulmonary artery, tibial artery, aorta, main pulmonary artery, elastic arteries, plasma non-esterified fatty acid (NEFA).

It is well known that diabetes is a high risk factor in arteriosclerosis [1, 2]. Although it is generally thought that elevated levels of circulating lipids might be involved, the mechanism of development of arteriosclerosis in diabetes is still unknown.

Our previous light microscope histochemical studies showed a rapid accumulation of triacylglycerol in branches of the pulmonary artery in diabetic rats, developing within three days of the injection of streptozotocin [3] and disappearing rapidly with insulin therapy [4].

The present study was initiated: 1) to determine the ultrastructural aspects of lipid deposits in pulmonary arteries during short-term experimental diabetes; 2) to search for possible lipid deposits in other arteries, both muscular and elastic; 3) to establish whether there was any relation between lipid deposition and circulating levels of insulin, glucose or lipids.

Material and Methods

Animals and Induction of Diabetes

Male Sprague-Dawley rats weighing 300–350 g were used. The series consisted of 24 diabetic and 17 normal control rats. The animals were fed a normal chow ad libitum (carbohydrate 53%, protein 21%, fat 4.5%, fibre 3% and required vitamins and minerals, obtained from Hankkija, Turku, Finland).

Diabetes was induced under pentobarbitone anaesthesia by an intracardiac [5] injection of streptozotocin (Upjohn, lot 1614 E, MCM 3), 65 mg/kg of body weight, as a freshly prepared 3% (w/v) solution in 0.1 mol/l citrate buffer (pH 4.5). No insulin treatment was given. The control rats received an equal volume of buffer. The diabetic rats were killed four days after the injection. All animals were weighed at the start and finish of the experiment, and checked for glycosuria (Clinitest, Ames, Elkhart, Indiana) and ketonuria (Ketostix, Ames).

Blood Sampling and Analysis

Blood was drawn from the retro-orbital venous plexus of the non-fasting rats through a capillary tube while the rat was under pentobarbitone anaesthesia, and collected in ice-cold EDTA tubes (final concentration of EDTA, 21.7 μmol/l) [6]. The animals were then killed by decapitation immediately.

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Table 1. Plasma glucose, insulin, NEFA, triacylglycerol and total cholesterol concentrations in normal and diabetic rats. (Mean ± SEM except for plasma NEFA values, which are given as median and range because of skew distribution)

Group	Number of animals	Plasma concentration				
		Glucose mg/100 ml	Insulin mU/l	NEFA mmol/l	Triacyl-glycerol mg/100 ml	Total cholesterol mg/100 ml
Normal control	17	101 ± 3	28.5 ± 2.3	0.5 (0.3–0.6)	161 ± 7	65 ± 3
Diabetic without arterial lipid	15	305 ± 8[a]	7.5 ± 1.0[b]	0.9[c] (0.8–1.1)	988 ± 128	129 ± 9
Diabetic with arterial lipid	9	334 ± 10[a]	5.1 ± 0.7[b]	1.3[c] (1.1–2.4)	1010 ± 131	121 ± 9

Blood was drawn from diabetic rats 4 days after injection with streptozotocin, 65 mg/kg
All animals had access to food and water until blood sampling under anaesthesia
[a,b] $p < 0.02$
[c] $p < 0.01$

Blood samples were cooled to 4 °C, and the plasma separated by centrifugation within 2 h. Plasma specimens were stored at −20 °C until analysed for glucose [7, 8], non-esterified fatty acids (NEFA) [9], triacylglycerol [10] and total cholesterol [11]. Plasma insulin was measured by radioimmunoassay (Phadebas Insulin Test, Pharmacia Diagnostics) with a standard of lyophilized porcine insulin, calibrated against the WHO insulin standard (WHO International Laboratory for Biological Standards). The detection limit of the assay was 3.0 mU/l. There was full cross-reactivity between porcine and rat insulins. All measurements of plasma constituents were made in duplicate.

Preparation of Tissue Specimens for Morphological Study

The pulmonary artery at 5 mm distal to hilus, the anterior tibial artery, and the proximal coronary artery are muscular arteries in the rat, having a thick media rich in smooth muscle cells. The aorta and the main pulmonary artery are elastic arteries consisting largely of elastic tissue.

Segments 2 mm long were taken from the following arteries immediately after decapitation: distal tibial, proximal part of left descending coronary, and pulmonary artery 5 mm distal to the hilus. Additional segments were taken from the ascending arch of the aorta and the main pulmonary artery. Segments were divided into strata from which eight pieces, 1 mm × 1 mm, were picked by random sampling [12], and placed in individual vials, fixed in 3% glutaraldehyde in 0.1 mol/l sodium cacodylate solution (pH 7.4), and washed in 0.1 mol/l sodium cacodylate (pH 7.4). The tissue specimens were then cut into smaller pieces (0.5 mm × 0.5 mm), and postfixed in 2% (w/v) OsO_4 in 0.1 mol/l cacodylate solution (pH 7.4). All steps were carried out at < 4 °C. Tissue specimens were dehydrated rapidly in graded acetone as described previously [13]. Eight pieces were then taken from each vial [12], and infiltrated with Epon [13]. Two sections of thickness 1 µm were cut from each block using a LKB Ultratome III and glass knives, and stained with toluidine blue for light microscopy. Two thin sections were also cut from each block, stained with uranyl acetate and lead citrate [14] and examined in a JEM 100 B electron microscope (Japan Electron Optics Laboratory, Japan).

Sixteen thin sections of each artery were thus examined in the electron microscope to determine the distribution and amount of lipid deposits in the arterial wall. The sections were primarily examined by one investigator and re-evaluated by one co-worker. The electron microscopic examination was performed without any knowledge whether an animal was diabetic or non-diabetic. The criterion for lipid was electron-opacity of oval or spherical structures, some of which were lamellar [13]. The amount of lipid in the arterial wall was graded from 0 to IV (very abundant). Special effort was made to distinguish accurately between grades 0 (no lipid) and I (slight lipid deposition).

Every section contained all the layers of the vessel wall. Smooth muscle cells were identified by myofilaments occurring in poorly defined bundles. Pinocytic vesicles were associated with the sarcolemma which was coated by basal membrane [15]. Foam cells were interpreted as smooth muscle cells, because some myofilaments were found in their cytoplasm.

Specimens were also taken from each artery for light microscope histochemical studies. These specimens were frozen with liquid N_2 and stored at −70 °C until analysed for triacylglycerol [16], cholesterol [17] and phospholipids [18]. The histochemical methods are specific [16–18].

Statistical Analysis

The results on plasma glucose, insulin, triacylglycerol and total cholesterol are given as mean ± SEM, and those on plasma NEFA as median and range because of a skew distribution. The differences between diabetic rats with and without arterial lipid was tested for plasma substances by Student's t test, except for plasma NEFA when the Mann-Whitney U test was used. The coefficient of variation of our NEFA assay was < 2%.

Results

Severity of Diabetes

The rats injected with streptozotocin lost 10–19% body weight and developed 3–5% glucosuria (Clinitest) and moderate (++) to severe (+++) ketonuria (Ketostix) in 4 days. They also had high plasma concentrations of glucose, NEFA, triacylglycerol and total cholesterol, and a low plasma concentration of insulin, confirming that they were severely diabetic at 4 days (Table 1).

Table 2. Relationship between plasma NEFA concentration and amount of lipid in muscular arteries of rats

Group (n)	Rat no.	Plasma NEFA mmol/l	Amount of lipid in muscular arteries[a]		
			Pulmonary	Coronary	Tibial
Normal [17]	–	0.3–0.6	0	0	0
Diabetic without arterial lipid [15]	–	0.8–1.1	0	0	0
Diabetic with arterial lipid [9]		1.1–2.4			
	1	1.1	II	I	I
	2	1.1	II	I	I
	3	1.2	II	I	II
	4	1.3	III	II	II
	5	1.3	II	II	II
	6	1.5	III	II	II
	7	1.6	III	II	II
	8	2.0	IV	II	III
	9	2.4	IV	III	III

[a] Amount of lipid graded from 0 to IV (very abundant). NEFA concentrations are given as range for each group

Accumulation of Lipid in the Muscular Arteries

Spherical or oval structures, from 0.04 μm to 0.19 μm in size were found in the muscular pulmonary, tibial and coronary arteries of 9 out of 24 diabetic rats, but in none of the normal control rats (Table 2). The lipid was located in medial smooth muscle cells, especially those in the inner media (Fig. 1). No lipid was seen in the endothelium, subendothelial space, or extracellular space of the media. Lipid was most abundant in the muscular pulmonary artery (Table 2). Lipid deposits were often seen near mitochondria and the endoplasmic reticulum (Figs. 1 and 2). Transformation of smooth muscle cells into foam cells (Fig. 3), with massive accumulation of lipid in the cells, was found in the muscular pulmonary artery of one diabetic rat (Table 2, rat 9).

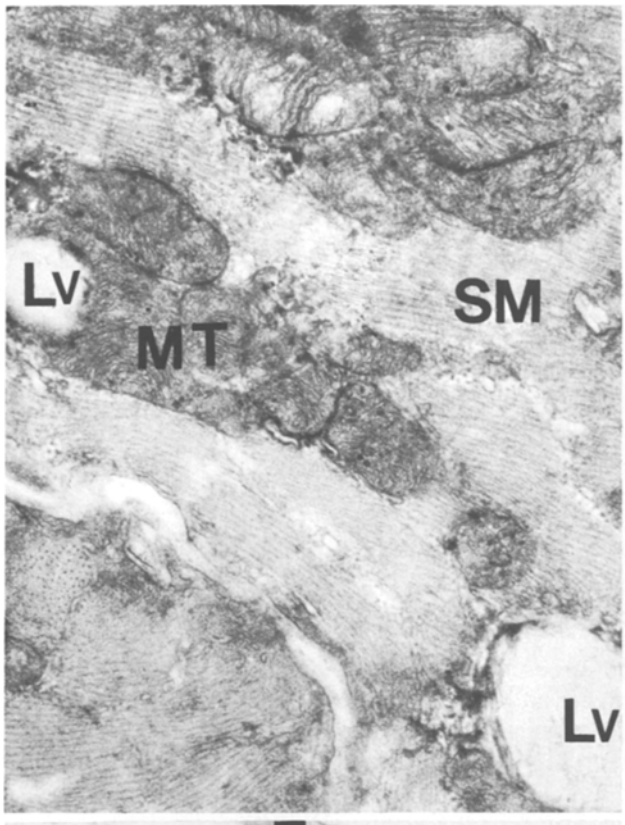
Light microscope histochemical studies demonstrated that the lipid deposits in the smooth muscle cells of the muscular arteries were triacylglycerol (Fig. 4). The histochemical reaction for triacylglycerol was negative in the muscular arteries of both diabetic rats without electron microscopically detectable lipid deposits and normal control rats. The histochemical reactions for lipid were also negative in the elastic arteries of all groups of rats. Neither cholesterol nor phospholipids were found histochemically in any arteries of the diabetic or control rats.

Relationship between Plasma NEFA Concentration and Amount of Lipid Deposited in Muscular Arteries

A comparison of the plasma concentrations of glucose, insulin and various lipids in the diabetic rats with and without arterial lipid deposits is given in Table 1. The primary differences between the two groups were that plasma NEFA concentrations were higher and insulin concentrations slightly lower in the diabetic rats with arterial deposits. The data presented in Table 2 show a relationship between the plasma NEFA concentration and the amount of lipid deposited in the arteries. No correlation was found between plasma NEFA and triacylglycerol concentrations in the diabetic rats with or without arterial lipid deposits.

Discussion

Our findings demonstrate a marked effect of acute diabetes on lipid metabolism in muscular arteries, resulting in an accumulation of triacylglycerol in medial smooth muscle cells. The deposits were often associated with the smooth endoplasmic reticulum (SER) found in the cells principally in tubular and vesicular forms [19]. The most prominent role for the SER is in the synthesis of lipids, for example triacylglycerol from glycerol and NEFA [19]. The uptake of NEFA by cells has been reported to be an energy independent process, which functions only to maintain an equilibrium between the plasma and cellular pools of NEFA [20]. On the other hand, the excess of long chain fatty acids may act as an uncoupling agent in the cell causing mitochondria to respire but not synthesize adenosine triphosphate (ATP) [21]. The positive relationship between the plasma NEFA concentration and the amount of lipid deposited in the muscular arteries (Table 2) suggests that lipid deposition could result from an increased uptake and esterification of plasma NEFA derived from adipose



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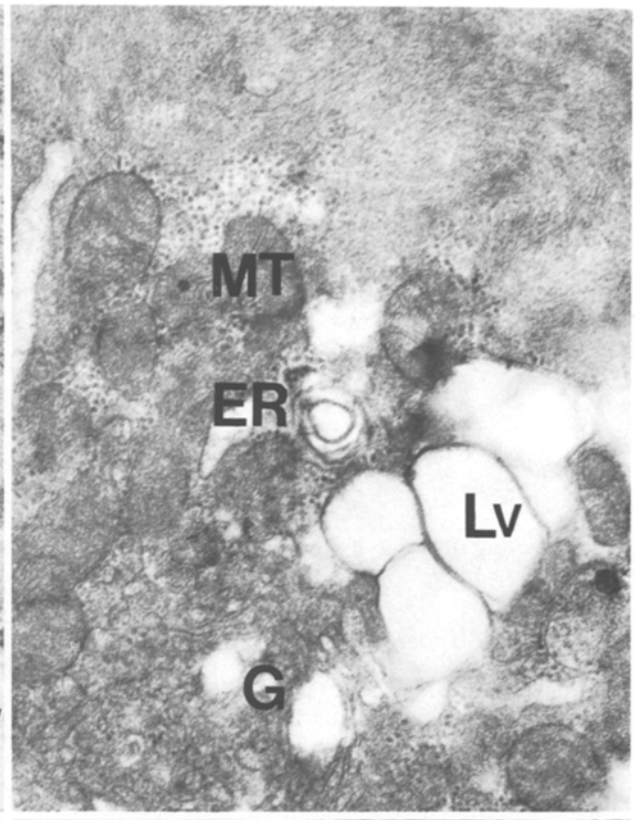

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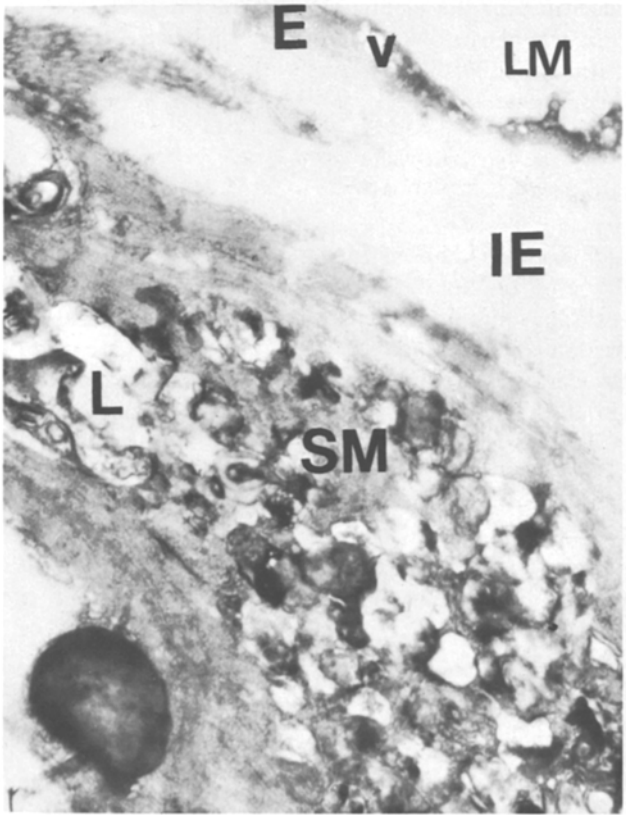

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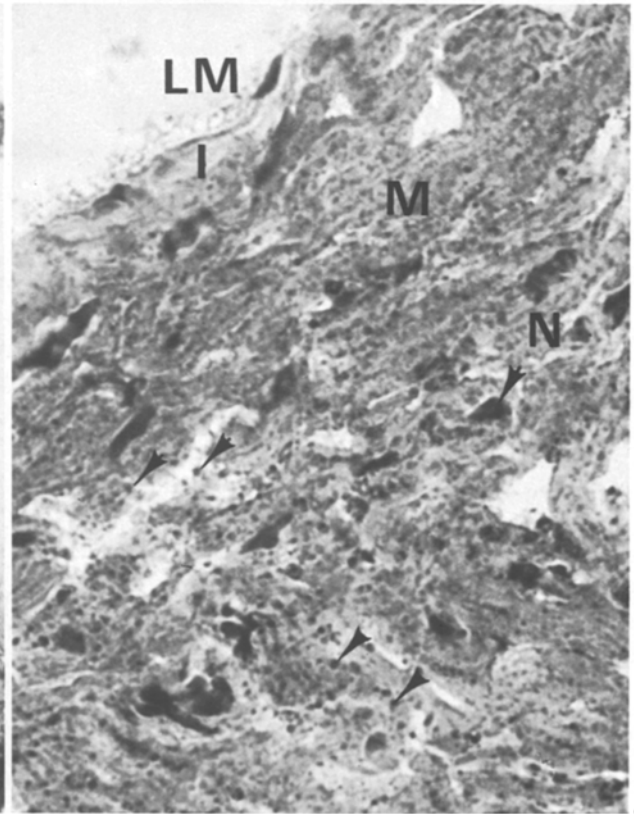

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tissue during insulin deficiency [22]. There may also be decreased utilization of NEFA by mitochondria [21].

Most NEFA in plasma is bound to albumin [23, 24, 25]. The molar ratio of NEFA to albumin in Plasma seldom exceeds 2 under normal conditions, but it can be 3.5 in severe diabetes [23, 26, 27]. It has been shown that the uptake of NEFA by perfused organs [28] and incubated cells [27, 29] is a function of the molar ratio NEFA to albumin in the medium, and that the uptake by incubated cells is greatly enhanced, sometimes producing injurious effects, when the ratio exceeds 3 [27, 29]. In the present study, assuming that the plasma albumin concentration was 0.48 mmol/l (3.2 g/100 ml) [30], the molar ratio of NEFA to albumin was in the range 0.7–1.2 in the normal rats, 1.6–2.4 in the diabetic rats without arterial lipid deposits, and 2.3–5.1 in the rats with arterial deposits.

The elevation of plasma NEFA concentrations is a characteristic feature of insulin deficiency [22], being proportional in degree to the severity of diabetes [31]. Thus the difference in plasma NEFA concentrations between our diabetic rats with and without arterial deposits may indicate a more severe diabetic state in the former group.

Many studies have shown premature arteriosclerosis in diabetics, especially in the muscular arteries of the leg [32, 33] and the coronary arteries [34, 35]. Even though our findings involve only short-term effects of diabetes on arterial lipid metabolism, they support the findings of others that muscular arteries are more frequently involved than elastic arteries in diabetes.

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Fig. 1. Muscular pulmonary artery section from a diabetic rat showing lipid containing vacuoles (Lv) in a medial smooth muscle cell (SM). The vacuoles are closely associated with mitochondria (MT). × 67000

Fig. 2. Coronary artery section from a diabetic rat showing accumulation of lipid (Lv) in a medial smooth muscle cell. The vacuoles (Lv) are associated with the endoplasmic reticulum (ER), Golgi apparatus (G) and mitochondria (MT). × 40000

Fig. 3. Muscular pulmonary artery from a diabetic rat with a very high plasma NEFA concentration (2.4 mmol/l), showing massive accumulation of lipid in a medial smooth muscle cell (SM). The lipid droplets (L) are spread throughout the cell. E = endothelium, IE = internal elastic lamina, LM = lumen, V = vesicles in the endothelium. × 15000

Fig. 4. Histochemical reactions in a muscular pulmonary artery from a diabetic rat demonstrating the presence of triacylglycerol deposits, seen as dark spots (arrows), throughout the media (M). The intima (I) does not contain lipid deposits. LM = lumen, N = nucleus. The tissue specimen was incubated with pancreatic lipase and then treated with Pb^{++} and S^{--} [16]. × 650

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