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The Liver as the Site of Carnitine Biosynthesis in Sheep with Alloxan-induced Diabetes

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Abstract

The total acid-soluble carnitine concentration in the livers of three sheep increased 20-fold, to 4530 nmol/g wet weight, 10–14 days after induction of the diabetic state by alloxan. There was a threefold increase in the total carnitine concentration of the kidney cortex and no significant change in that of heart or skeletal muscle (M. biceps femoris).

In normal animals no significant difference was observed between the carnitine concentrations of blood taken from indwelling hepatic and portal cannulae. However, 10–14 days after administration of alloxan there was a substantial increase in the carnitine concentration of both portal and hepatic venous blood in most animals. An hepatic–portal difference in carnitine concentration became apparent only when the daily food intakes of the animals dropped to a third or less of the normal value, at which stage there was a net production of carnitine by the liver. No significant difference was observed between the carnitine concentrations of femoral arterial and femoral venous blood samples in either the normal or the alloxan-induced diabetic state.

The results indicate that the increased amounts of carnitine observed in the circulating blood and in the livers of sheep in the terminal diabetic state are not due to mobilization of carnitine from the muscle tissues but result from increased synthesis of carnitine within the liver itself.

Introduction

Previous work has shown that the total carnitine concentration in sheep liver increases in starvation (Snoswell and Henderson 1970) and in alloxan-induced diabetes (Snoswell and Koundakjian 1972). This is in direct contrast to the situation in the rat, where the total concentration of carnitine in liver remains essentially the same in the normal and diabetic states (Pearson and Tubbs 1967), and also to that in the guinea pig, where the sum of acetyl- and free carnitine falls during diabetes (Erfle and Sauer 1967). Also, alloxan-induced diabetes in rats leads to a decrease of about 50% in the carnitine concentration in the muscle tissues and in the total body pool (Mehlman *et al.* 1969).

It has been suggested that the increases in liver carnitine concentration in starved and diabetic sheep may indicate increased biosynthesis of this compound in the liver (Snoswell and Henderson 1970). However, in view of the very high concentrations of carnitine in muscle tissues of sheep (Snoswell and Koundakjian 1972), the possibility arises that the accumulation of carnitine in the liver could be due to mobilization of carnitine from the muscle tissues in times of metabolic stress. The aim of this investigation was to determine which tissue was the source of the carnitine that accumulates in the liver during alloxan-induced diabetes.

Sheep were surgically prepared with indwelling portal and hepatic venous cannulae and femoral venous and arterial cannulae in order to examine differences in blood

carnitine concentrations across the liver and hind limb during development of diabetes following administration of alloxan.

Materials and Methods

Animals

Merino wethers weighing 30–35 kg were used. They were kept in metabolism cages and were provided with water *ad libitum* and a daily ration of 750 g wheaten hay chaff–250 g lucerne chaff or 1000 g lucerne chaff. Prior to surgery the animals were fasted for 48 h.

Surgical Cannulation of Hepatic and Portal Veins

Two methods have been reported for placing cannulae into portal and hepatic veins of sheep for repeated sampling in the conscious animal (Harrison 1969; Katz and Bergman 1969). In the present study difficulties were experienced in obtaining workable preparations with either method. Therefore an improvement of the method described by Katz and Bergman (1969) was developed.

Anaesthesia was induced with thiopental sodium (25 mg/kg body weight) and maintained with Fluothane and oxygen. The sheep was laid on its left side, clipped and sterilized with nitromersol (5 mg/ml) over the right flank and thorax. Access was gained to the liver via a paracostal incision 3–4 cm behind the last rib and extending from the sternum to the midflank region. The ventral lobe of the liver was retracted from the diaphragm to expose the posterior vena cava where it passes through the diaphragm. The entrance of the hepatic vein to the vena cava could be palpated through the wall of the vena cava and the hepatic vein was followed as it passed centrally down the ventral lobe of the liver. The hepatic vein was cannulated from the visceral surface about 5 cm from the ventral border by making a stab incision with a large-bore hypodermic needle attached to a suitable handle. The bevel of the needle faced towards the liver surface. A polyvinyl tube (1·5 mm i.d., 2·5 mm o.d. and 60 cm in length) was passed through the lumen of the needle into the vein and the needle withdrawn while keeping the tube in place. Correct positioning of the tube at this stage was indicated by a free flow of blood from the tube. The tube was then passed further into the hepatic vein until it could be palpated within the lumen of the vena cava. The tip was subsequently withdrawn into the hepatic vein until it was about 2 cm from the vena cava (as determined by palpation). The tube was then fixed to the surface of the liver with tissue adhesive (methyl α-cyanoacrylate) and exteriorized.

The portal vein was cannulated near its entry to the liver by means of a stab incision, after a purse-string suture had first been placed in position to stop subsequent haemorrhaging. A similar cannula to that used for the hepatic vein was then passed 4–5 cm towards the liver and fixed in position with tissue adhesive. This cannula was exteriorized, attached to the skin with sutures (size 0 braided silk) and taken up onto the back of the sheep for ease of sampling.

The operation was completed in 1·5 h and the sheep was returned to its cage. The animals normally took 4–5 days to return to full food intake and then daily blood sampling was commenced. The cannulae were kept patent by flushing daily with heparin–saline mixture (100 units heparin/ml).

Following the intravenous (femoral venous) infusion of sulphobromophthalein sodium, blood samples taken from the hepatic venous cannulae showed very low concentrations of the dye compared with those taken from the portal cannulae. This indicated that an effective removal of the dye by the liver had occurred, and that the hepatic and portal cannulae had been correctly placed. The positions of the cannulae were also checked by post-mortem examination and in all cases were found to be correct.

Cannulation of the Femoral Arteries and Veins

Polyvinyl cannulae were inserted into the femoral vein and artery via the saphenous branches of the vessels. Access to the vessels was gained via a skin incision on the inside of the hind limb near the pelvic symphysis. The artery and vein were dissected free from the surrounding tissues and ligated. Cannulae (1·0 mm i.d., 1·5 mm o.d.) were introduced through incisions and passed for several centimetres up into the deep femoral vessels. The cannulae were tied in place with ligatures, the skin incision closed with metal clips and the cannulae taken up behind the leg and tied to the wool on the back of the sheep. The cannulae were kept patent by flushing with heparin–saline mixture (100 units heparin/ml) daily.

Induction of Diabetes with Alloxan

The sheep were allowed to stabilize on full food intake for 5–7 days after surgery. Blood samples were then taken on several consecutive days to establish normal values, after which alloxan (Koch-Light Ltd., 65 mg/kg body weight) was dissolved in sterile saline (0·9%) and immediately injected into a jugular vein.

Blood glucose, as monitored with Dextrostix (Ames Pharmaceuticals Ltd), rose to approximately 180 mg/100 ml within 2 days of alloxan administration and total blood ketones (acetoacetate plus 3-hydroxybutyrate) rose to 5–7 mM within 3 days. After approximately 7 days most of the diabetic sheep spontaneously decreased their food intakes and in the terminal stages (10–14 days after alloxan administration) these animals were eating a third of their normal intakes or less. The animals were slaughtered on the day the food intakes fell to zero.

Some animals did not reduce their food intakes in this manner and these animals have not been included in the main body of observations reported here. However, they were used to check whether the deprivation of food had any influence on carnitine metabolism. This variable response of sheep to alloxan-induced diabetes may be related to the degree of acidosis associated with the ketosis. In the present study the animals that reduced their food intakes spontaneously all showed acidosis as judged by the low pH of urine samples.

Measurement of Free Carnitine and Carnitine Esters

Total acid-soluble carnitine in the neutralized perchloric acid extracts of the blood samples following alkaline hydrolysis was measured using the method described by Marquis and Fritz (1964) for free carnitine.

Tissue samples and perchloric acid extracts of these were prepared as described previously (Snoswell and Henderson 1970). Acetylcarnitine was estimated by the method of Pearson and Tubbs (1964) and total acid-soluble and acid-insoluble carnitine were measured by the method of Pearson and Tubbs (1967).

Chemicals

L-Carnitine hydrochloride and *O*-acetyl-L-carnitine hydrochloride were generously supplied by Otsuka Pharmaceuticals, Japan.

Results

Tissue Carnitine Concentrations

The results presented in Table 1 show a 20-fold increase in total acid-soluble carnitine and a 30-fold increase in acetylcarnitine in livers of sheep with severe alloxan-induced diabetes. These increases are much more pronounced than those previously reported for sheep 3 days after alloxan administration (Snoswell and Koundakjian 1972) and appear in the present work to be correlated with the drastically reduced food intakes in the later stages of the diabetic state. Despite the fact that the livers of the diabetic sheep were extremely fatty, the acid-insoluble carnitine fraction (not shown in the table), which contains the long-chain fatty acylcarnitine esters, constituted less than 2% of the total tissue carnitine, while acetylcarnitine contributed two-thirds of the total carnitine.

The total carnitine concentration in the kidney cortex increased threefold in the diabetic animals (Table 1). This result is probably related to the raised blood concentration of carnitine and increased clearance of carnitine into the urine. In sheep Al-567 the total output of carnitine in the urine on the day prior to slaughter was 1600 μmol and on the previous day 1500 μmol. The daily output of carnitine in the urine of normal sheep maintained under comparable conditions was 73 ± 13 μmol/day (mean ± S.E.M. for nine observations on three animals).

No significant changes in the total carnitine concentration of muscle tissue (heart or skeletal) were observed in the diabetic animals (Table 1).

Blood Carnitine Concentrations

The values shown in Table 2 indicate marked hepatic–portal differences in the concentrations of carnitine in all sheep in the latter stages of the diabetic state and this is particularly pronounced in sheep no. Al-761. This hepatic–portal difference did not become apparent until 9–10 days after the administration of alloxan, i.e. until the food intake dropped to a third or less of the normal daily intake. Data for the daily blood samples up to 9 days after alloxan administration are not included in Table 2, as no hepatic–portal difference was apparent. No significant difference was observed

Table 1. Free carnitine and carnitine ester concentrations in tissues of normal sheep and sheep with alloxan-induced diabetes

Tissues from three Merino wethers were immediately frozen with aluminium-faced tongs previously cooled in liquid nitrogen and the frozen powders were extracted with $HClO_4$ and assayed as described in the text. The normal liver samples were removed within 2 min of the administration of thiopental sodium at the time of surgery. The sheep were subsequently made diabetic by the intravenous injection of alloxan (65 mg/kg body wt). The animals were slaughtered 10–14 days later and the tissues immediately freeze-clamped as above. The normal kidney, heart and skeletal muscle samples were obtained from another group of four Merino wethers maintained under similar conditions, as reported previously (Snoswell and Koundakjian 1972). Concentrations, given as means ± S.E.M., are expressed as nmol/g wet wt

Tissue	State	Acetyl-carnitine	Free carnitine	Total acid-soluble carnitine
Liver	Normal	98±46	79±11	227±59
	Diabetic	3090±340	797±184	4530±610
Kidney cortex	Normal	67±18	415±45	538±64
	Diabetic	999±32	438±153	1740±432
Heart	Normal	812±83	2060±323	3510±143
	Diabetic	1150±115	1740±275	3830±217
Skeletal muscle (M. biceps femoris)	Normal	1820±478	9860±1380	12900±880
	Diabetic	12000±2460	3240±1190	17100±2600

between the carnitine concentrations of hepatic and portal blood in normal animals: the hepatic concentration was 36·1 ± 1·8 nmol/ml and the portal concentration was 35·3 ± 1·7 nmol/ml (means ± S.E.M. of 23 observations on nine animals).

There was no significant difference in the carnitine concentration of femoral venous compared with femoral arterial blood in either the normal or diabetic state. In the normal state the femoral arterial concentration was 36·4 ± 2·5 nmol/ml and the femoral venous concentration was 36·3 ± 2·5 nmol/ml (means ± S.E.M. of 16 observations on five animals); in the terminal stages of the diabetic state the femoral arterial concentration was 104 ± 7·6 nmol/ml, and the femoral venous concentration was 102 ± 5·6 nmol/ml (means ± S.E.M. of 10 observations on three animals). At this stage the portal carnitine concentration was equal to that of the femoral artery.

The pronounced increases in the concentration of carnitine in the blood (particularly hepatic blood) after administration of alloxan (Table 2) only occurred after appreciable spontaneous reductions in food intake in the latter stages of diabetes. This effect was not due to starvation *per se*, as normal sheep completely deprived of food for 7 days showed no significant increase in blood carnitine concentration, nor was any hepatic–portal difference in blood carnitine concentration observed. Similarly,

sheep that had been made diabetic with alloxan and that did not decrease their food intake within 14 days of alloxan administration were subsequently deprived of food for a 7-day period; these sheep also failed to show any significant rise in blood carnitine concentration.

Table 2. Carnitine concentrations in portal and hepatic venous blood samples from normal sheep and sheep with alloxan-induced diabetes

Blood samples from normal sheep were collected daily prior to alloxan administration and those from diabetic sheep were collected 10–14 days after the intravenous administration of alloxan (65 mg/kg body wt). The samples were collected simultaneously from indwelling hepatic and portal cannulae. Duplicate 5-ml aliquots were immediately mixed with an equal volume of 15% $HClO_4$ and total acid-soluble carnitine was estimated as described in the Methods. The values for each duplicate assay are recorded; n.d., not determined

Sheep	Sample	Blood carnitine concentrations (nmol/ml)						
		Normal sheep			Diabetic sheep			
		Successive daily samples:			Days prior to slaughter:			
		1	2	3	3	2	1	0
A1-761	Hepatic	21,22	34,32	34,33	98,97	70,68	59,59	279,273
	Portal	24,22	38,34	34,32	38,34	23,21	37,35	201,193
A1-567	Hepatic	45,46	51,46	40,39	n.d.	78,76	60,56	108,102
	Portal	44,44	48,43	42,40	n.d.	70,69	45,43	95,94
A1-742	Hepatic	n.d.	n.d.	46,42	n.d.	n.d.	123,128	138,135
	Portal	n.d.	n.d.	47,45	n.d.	n.d.	115,111	116,116

Discussion

The results presented here indicate that even in the terminal stages of alloxan-induced diabetes in sheep there is no reduction in the concentration of carnitine in the muscle tissues as is seen in the alloxan-diabetic rat (Mehlman *et al.* 1969). Thus, it is unlikely that the accumulation of carnitine in the livers of diabetic sheep is due to mobilization from muscle tissues. Further, in the terminal stages of the diabetic state, the concentration of carnitine in the circulating blood increased, as did the excretion of carnitine in the urine. At the same time, an appreciable hepatic–portal difference in the concentration of carnitine in the blood was observed. As the concentration of carnitine in the arterial blood at that stage was equal to that in the portal blood, the apparent production by the liver could not have been due to carnitine production in the gut or a contribution to the hepatic venous output from the hepatic artery. Thus, the observed result must have been due to production of carnitine in the liver.

The marked hepatic–portal differences in blood carnitine concentrations were only seen in the terminal stages of the diabetic state and were probably due to reduced blood flow rates through the liver at that stage. The spontaneous reduction in food intake seemed to be an essential feature of this phenomenon, as the deliberate withholding of food from diabetic sheep that did not spontaneously reduce their food intakes failed to produce any significant change in blood carnitine concentrations. Although the physiological basis of the spontaneous reduction in food intake in the terminal stages of the diabetic state is unclear, this phenomenon appears to provide the metabolic inducement for the increased synthesis of carnitine by sheep liver. The synthesis of carnitine at this stage is probably related to the acetyl-buffering action of

carnitine and carnitine acetyltransferase (see Snoswell and Koundakjian 1972 for discussion), as the bulk of carnitine in the liver in the terminal stages of the diabetic state is in the form of acetylcarnitine.

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References

Erfle, J. D., and Sauer, F. (1967). Acetyl coenzyme A and acetylcarnitine concentration and turnover rates in muscle and liver of the ketotic rat and guinea pig. *J. Biol. Chem.* **242**, 1988–96.

Harrison, F. A. (1969). The introduction and maintenance of permanently indwelling catheters in portal and hepatic veins of the sheep. *J. Physiol.* **200**, 28–30P.

Katz, M. L., and Bergman, E. N. (1969). A method for the simultaneous cannulation of the major splanchnic blood vessels of the sheep. *Am. J. Vet. Res.* **30**, 655–61.

Marquis, N. R., and Fritz, I. B. (1964). Enzymological determination of free carnitine concentrations in rat tissues. *J. Lipid Res.* **5**, 184–7.

Mehlman, M. A., Abdel-Kader, M., and Therriault, D. G. (1969). Metabolism turnover time, half life and body pool of carnitine-^{14}C in normal, alloxan diabetic and insulin treated rats. *Life Sci.* **8**, 465–72.

Pearson, D. J., and Tubbs, P. K. (1964). Acetylcarnitine in heart and liver. *Nature (Lond.)* **202**, 91.

Pearson, D. J., and Tubbs, P. K. (1967). Carnitine derivatives in rat tissues. *Biochem. J.* **105**, 953–63.

Snoswell, A. M., and Henderson, G. D. (1970). Aspects of carnitine ester metabolism in sheep liver. *Biochem. J.* **119**, 59–65.

Snoswell, A. M., and Koundakjian, P. P. (1972). Relationships between carnitine and coenzyme A esters in tissues of normal and alloxan-diabetic sheep. *Biochem. J.* **127**, 133–41.

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