The Effect of Diabetes on Mevalonate Metabolism in the Rat

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Summary. Previous studies have demonstrated a major difference in mevalonate metabolism by male and female rats by both the shunt and sterol pathways. This important sex difference was shown to be related to the presence of oestrogens and/or progesterone. In the present study we have investigated the effects of streptozotocin-induced diabetes on mevalonate metabolism. Firstly, insulin deficiency decreased the ability of the rats to oxidize mevalonate to carbon dioxide by the shunt pathway both in vivo and in vitro. This decrease was reversed by insulin treatment both in the intact animals as well as in tissue slices. Secondly, diabetes caused a marked increase in hepatic sterologenesis in the intact animal. This is the first demonstration that insulin plays a significant regulatory role in both the shunt and sterol pathways of mevalonate metabolism.

Key words: Diabetes, cholesterogenesis, mevalonate, insulin, mevalonate shunt, sterologenesis.

tabolize circulating mevalonate in vivo by the sterol pathway at 20 times the rate of any other tissue [5]. We have also demonstrated that the kidney, both in vivo and in vitro, accounts for more than half of the total mevalonate oxidized to CO₂ by the shunt pathway [3, 6, 7]. Similar results have been reported from other laboratories [8, 9].

Recent reports from this laboratory [3, 4, 10] have demonstrated further that the female shunts mevalonate away from the sterol pathway at twice the rate of the male. This important sex difference was shown to result from the hormonal control of mevalonate metabolism by oestrogen and/or progesterone. The finding of sex hormonal regulation of mevalonate metabolism has stimulated studies to determine whether the mevalonate pathways are influenced by other hormonal factors. This study was designed to investigate the effect of diabetes on the metabolism of mevalonic acid to sterols and carbon dioxide in intact animals and in tissue slices.

Materials and Methods

Materials

Materials were obtained as follows: (RS)-[5-¹⁴C]mevalonic acid, sodium salt (15 mCi/mmol) from Research Products, Elk Grove, Illinois, USA; unlabelled (RS)-mevalonolactone from Sigma Chemicals, and streptozotocin from Sigma Chemicals, St. Louis, Missouri, USA and from Upjohn, Kalamazoo, Michigan, USA; U-100 insulin from Eli Lilly, Indianapolis, Indiana, USA; thin layer polygram SilG plates from Brinkmann Instruments, Westbury, New York. Radio-autography was carried out on Kodak RP-14 X-ray film (Eastman Kodak, Rochester, New York). The liquid scintillation fluid contained scintillation-grade toluene obtained from Packard Corporation, Downers Grove, Illinois, USA; 1- α , 2- α tritiated cholesterol (43 Ci/mmol) and scintillation-grade PPO and POPOP were purchased from Amersham/Searle, Arlington Heights, Illinois, USA; and Bio-Solv III and Triton X-100 from Beckman Instruments, Palo Alto, California. The Alzet pumps were purchased from Alza, Palo Alto, California.

The synthesis of cholesterol de novo in man and other mammals is regulated primarily by the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, the product of which is mevalonate. Mevalonate can be metabolized by two major mechanisms. Firstly, it can be converted by the sterol pathway to ubiquinone, dolichol and cholesterol; and secondly, mevalonate can be metabolized by a non-sterol (shunt) pathway which ultimately oxidizes it to carbon dioxide [1, 2]. This alternative pathway, which diverts mevalonate from cholesterol production, can account for as much as 32% of the circulating mevalonate metabolized in vivo [3, 4].

In previous studies we have shown that physiological levels of circulating mevalonate are metabolized by both of these pathways primarily in the kidneys rather than in the liver. The kidney was found to me-

Table 1. Effect of diabetes and of insulin treatment on mevalonate oxidation in male rats

Mevalonate oxidation to CO ₂ (nmol/6h)		
Control (n=5)	Diabetic (n=6)	Insulin-treated (n=6)
54.8 ± 3.4	28.8 ± 2.1	58.9 ± 6.6

The values are expressed as mean ± SEM. Numbers in parentheses refer to the number of animals tested

Animals and Procedures

Experimental methods used have been described previously. [4, 7]. For studies in vivo, male Sprague-Dawley rats weighing between 200 and 400 g were maintained on a reverse 12-h light cycle for one week before the study and were fed Berkeley rat and mouse diet and water ad libitum. The animals were fasted for 24 h, after which they were injected IV with streptozotocin (125 mg/kg rat weight) dissolved in 0.1 mol/l citrate buffer (pH 4.5) [11]. Only animals that developed 4+ glycosuria were used in these studies; insulin-treated animals were given U-100 regular insulin (2–5 U/day) by IP injection or by SC Alzet pump implants at the earliest evidence of glycosuria. All in vivo experiments were initiated 5–6 days after the streptozotocin injection. The animals were anaesthetized with diethyl ether, and 136 µg of [5-¹⁴C]mevalonic acid in 0.4 ml of 0.9% NaCl was injected into the tail vein of each rat. For metabolic studies at approximately physiological levels, the concentration of [5-¹⁴C]mevalonic acid was reduced to 8.5 µg. Following injection the animals were placed in 2-l widemouth Erlenmeyer flasks fitted with two-hole stoppers. Air was drawn through the flasks at the rate of 2 l/min, and expired ¹⁴CO₂ was collected in 1 N NaOH solution (2 × 180 ml). At each hour, 0.2-ml samples of the NaOH solution were added to 10 ml of liquid scintillation fluid consisting of toluene (1000 ml), Beckman Bio-Solv III (300 ml), water (100 ml), and PPO (6 g). The amount of ¹⁴CO₂ collected was determined on either a Beckman LS-330 or LS-230 liquid scintillation counter. After 6 h the rats were killed and the livers, kidneys and carcasses were saponified separately and analyzed for ¹⁴C-lipids as described below.

For the studies in vitro, female Sprague-Dawley rats weighing 200–400 g were maintained routinely on Berkeley rat and mouse chow and water ad libitum. Streptozotocin injection and insulin treatment were the same as described earlier. The animals were anaesthetized with diethyl ether and decapitated, after which the kidneys were rapidly removed and placed in Krebs-Ringer phosphate buffer. A whole kidney was sliced into ribbons on a McIlwain tissue slicer and a 200-mg sample was placed in the outer well of a 25-ml centre well flask containing 2 ml of Krebs-Ringer phosphate buffer, 1 µCi of [5-¹⁴C]mevalonate and unlabelled mevalonate to yield a final concentration of 0.1 mmol/l. The flasks were stoppered with serum caps and incubated at 37 °C for 1 h in a Dubnoff metabolic shaker at 60 oscillations/min. All experiments were carried out in duplicate.

At the end of the incubation period, 1 N NaOH (0.9 ml) was injected through the serum caps into the centre wells, and 1 N H₂SO₄ (1 ml) was injected into each outer well. ¹⁴CO₂ was collected in the centre wells by re-incubating the samples for 15 min in the Dubnoff shaker. Aliquots (0.1 ml) of the centre well contents were added to 10 ml of Beckman Bio-Solv III scintillation counting fluid previously described. All values were corrected for small amounts of volatile ¹⁴C recovered from incubations with previously boiled tissues.

Non-saponifiable Lipids

After the addition of internal standards of tritiated cholesterol, the tissues to be studied were placed in Erlenmeyer flasks and refluxed overnight with 90% KOH:70% ethyl alcohol (1:5) as described

previously [4, 7]. The non-saponifiable material was extracted with petroleum ether (50 ml). The petroleum ether was dried and dissolved in chloroform (1 ml) and 100-µl samples were applied to thin-layer chromatography plates. The plates were developed in ethyl acetate:benzene (1:5) for 50 min, and radio-autographed on Kodak RP-14 X-ray film for 3–5 days. Areas of the plates corresponding to cholesterol, lanosterol and squalene markers were cut out and placed into vials containing 10 ml counting solution consisting of toluene (1.33 l) Triton X-100 (0.66 l), PPO (9.33 g) and POPOP (0.133 g). The gain and discriminator window settings of the scintillation counter were adjusted so that < 0.02% of the tritium counts were read in the ¹⁴C window and approximately 15% of the ¹⁴C counts were recorded in the tritium window. The amount of tritiated cholesterol added as internal standard was adjusted so that the tritium counts were approximately five times > the ¹⁴C counts. After correction for spillover of tritium and ¹⁴C and for background radioactivity, counts were expressed as d.p.m. Although (RS)-[5-¹⁴C]mevalonate was used in this study, it is well established that only the (R) isomer is metabolized [2]. For this reason all calculations assume that half of the administered mevalonate is inactive.

The histological sections, stained with haematoxylin and eosin, were prepared by standard techniques.

Results

Effect of Diabetes and Insulin Therapy on the Mevalonate Shunt Pathway

The initial experiments in vivo were concerned with determining the influence of diabetes and insulin therapy on the shunt pathway in the intact male animal. The metabolism of IV injected single doses of [5-¹⁴C]mevalonate (136 µg) to ¹⁴CO₂ was monitored for 6-h periods in five control animals and in 12 male diabetic rats, one-half of whom were treated with insulin (Table 1). Diabetes suppressed the shunt activity in the animals by approximately 50% ($p < 0.001$), but the activity was completely restored by insulin therapy (control versus treated diabetic rats $p > 0.1$).

Effects of Diabetes and Insulin Therapy on the Sterol Pathway

These effects were studied in the kidneys, liver and carcass of the same rats used above. As shown in Figure 1a, diabetes significantly influenced the accumulation of labelled sterols from ¹⁴C-mevalonate in the kidney. The average amount of mevalonate incorporated into sterols in the control male kidneys (136 nmol/6 h) was approximately twice that found in the kidneys of diabetic rats (74 nmol/6 h, $p < 0.01$). Insulin therapy did not significantly restore the synthesis of sterols in the kidney.

Diabetes caused an opposite change in hepatic sterols synthesized from circulating mevalonate (Figure 1b). The mean ¹⁴C-sterol synthesis in the diabetic male liver was twofold greater than that found in the control male liver (37 versus 18 nmol/6 h, $p < 0.01$).

Carcass sterols synthesized from circulating ¹⁴C-mevalonate increased in the diabetic rat (50 ± 4 ver-

Fig. 1 a–b. Conversion of circulating [5-¹⁴C]mevalonate in vivo to non-saponifiable lipids (squalene, lanosterol and cholesterol) in (a) the kidney and (b) the liver of male control (C), diabetic (D), and diabetic rats treated with insulin (D+I). The rats were those used in the experiments shown in Table 1. Bars indicate mean values $\pm$ SEM

Table 2. Metabolism of [5-¹⁴C]mevalonate in the liver of rats at physiological dose levels

Pathway	Control	Diabetic
Shunt (nmol CO ₂)	3.18	1.92
Liver non-saponifiable lipids (nmol)	1.12	2.53

Values represent mean values from duplicate animals

Table 3. [5-¹⁴C]mevalonate oxidation by rat kidney slices in vitro

Mevalonate oxidation to CO ₂ (nmol g ⁻¹ h ⁻¹)		
Control	Diabetic	Insulin-treated
24.1 $\pm$ 1.6	12.7 $\pm$ 0.5	28.9 $\pm$ 1.8

Each value represents the mean $\pm$ SEM of duplicate determinations on six female rats

sus 84 ± 9 nmol/6h; $p < 0.01$). Insulin treatment did not significantly influence sterol synthesis in the carcass (84 ± 9 versus 78 ± 5 nmol/6h).

The Effect of Substrate Concentration on Mevalonate Metabolism in Vivo by the Shunt and Sterol Pathways

Studies were next carried out to determine whether a difference in mevalonate pool size between the diabetic and normal animals could account for the observed differences in mevalonate metabolism in the diabetic animal. The amount of mevalonate in the extracellular pool of a 200-g rat has previously been calculated by this laboratory to be about 2.4 μ g [5], a figure that is similar to that of Popják et al. [14]. We

therefore studied the fate of mevalonate after injection of either 8.5 μ g/[5-¹⁴C]mevalonate, i.e. approximately a physiological dose, or 136 μ g/[5-¹⁴C]mevalonate – approximately 15 times the physiological level. As shown in Table 2, using physiological doses, diabetes reduced the shunt activity and increased sterol production in the liver, thus confirming the observations using higher levels of injected mevalonate (Table 1 and Fig. 1).

Metabolism of Mevalonate in Kidney Slices

Since the kidney is the primary site of mevalonate metabolism by the shunt mechanism [3, 7, 8], we evaluated the effect of diabetes on mevalonate metabolism by the kidney in vitro. As shown in Table 3, kidney slices from six normal animals metabolized [5-¹⁴C]mevalonate to ¹⁴CO₂ at approximately twice the rate of that found in kidney slices from diabetic rats ($p < 0.001$). Moreover, insulin treatment of the intact diabetic animals 2 days before study, completely restored the mevalonate shunt activity of the diabetic kidney.

Histological sections of the kidneys from the streptozotocin-treated rats showed minimal vacuolization of the tubular cells, with no evidence of interstitial changes. The glomeruli had no pathological changes. Insulin therapy appeared to decrease the tubular cell vacuolization slightly.

Discussion

The major finding of the present study is that insulin deficiency causes severe perturbations in both the sterol and shunt pathways of mevalonate metabolism. Streptozotocin diabetes markedly decreased the ability of the intact rat to divert mevalonate from sterol synthesis into the shunt pathway. The diabetic rat oxidized mevalonate to carbon dioxide by the shunt mechanism at only 50% of the normal rate.

Previous studies from this laboratory have demonstrated that the kidney is the primary site of mevalonate shunt activity in the rat [7]. When tissue slices were incubated with [5-¹⁴C]mevalonate, the insulin-deficient kidney had approximately 50% of the control shunt activity. While quantitative extrapolations from results in vitro to in vivo must be made with caution, it seems reasonable that the influence of diabetes upon the mevalonate shunt pathway may be ascribed to a direct effect of insulinopenia on the renal metabolism of mevalonate. The possibility that the reduced shunt activity was due to nonspecific streptozotocin induced kidney damage was ruled out by two observations. Firstly, only minimal histological changes were detected in the kidneys and secondly, insulin

treatment of the streptozotocin-diabetic rats caused a significant and rapid reversal of the depressed mevalonate shunt activity both in vivo and in vitro.

Shunt activity was consistently depressed in the diabetic rats, regardless of whether we administered approximately physiological or 15 times physiological levels of [^{14}C]mevalonate. In addition, the effect of diabetes on mevalonate shunt metabolism was demonstrated by experiments in vitro, where activity in kidney slices of the insulin treated diabetic animals was restored to normal. These observations suggest that differences in mevalonate pool size as well as alterations in intermediate pools cannot be responsible for the decreased mevalonate shunt activity noted in the diabetic rat.

Concomitant with its effects on the shunt pathway, insulin deficiency had a pronounced influence on the sterol pathway of mevalonate metabolism in that diabetes increased its incorporation into sterols by twofold in the liver. This increase was not always reversed by insulin. However, it is well known that insulin has stimulatory effects on de novo cholesterol synthesis [12, 13]. The mechanism by which diabetes consistently increased hepatic sterologenesi was not examined in the present study. However, we have demonstrated previously that removal of the kidneys, the major tissue site of shunt activity, causes a fivefold increase in the conversion of mevalonate to cholesterol in the liver. Thus, it is possible that when the renal shunt and sterol pathways are depressed by the diabetic state, mevalonate is channelled into the hepatic sterol pathway at an enhanced rate.

While the physiological significance of the shunt pathway is still unknown, we have demonstrated that mevalonate oxidation in normal human females is twice that of males [10]. Further, Fogelman et al. have shown that the shunt activity in a hypercholesterolaemic human male is approximately half that of a normal male [2]. Although the pathological implications of the increased hepatic conversion of mevalonate to sterols in diabetics have not yet been evaluated, this finding obviously correlates with the increased serum and liver cholesterol levels found in diabetic rats [15, 16] and man [17, 18]. Studies are currently in progress to determine whether alterations in the pathways of mevalonate metabolism, comparable to those observed in the rat, are likewise present in human diabetes.

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