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THE EFFECT OF DIABETES ON THE *IN VIVO* ACETYLATION CAPACITY OF THE SPONTANEOUSLY DIABETIC, INSULIN-DEPENDENT BB/EDINBURGH WISTAR RAT

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Abstract—In contrast to previous studies using chemically-induced diabetic rats, the *in vivo* acetylation of sulphamethazine is increased in spontaneously diabetic, insulin-dependent BB/Edinburgh (BB/E) Wistar rats compared to non-diabetic control animals from the same colony. In both diabetic and non-diabetic rats, male animals had a significantly higher acetylation capacity than female animals. The percentage recovery of the administered dose was significantly higher in urine samples from female rats.

N-Acetylation of drugs containing arylamine or hydrazino functions is catalysed by the group of enzymes known as *N*-acetyltransferases (EC 2.3.1.5) and involves acetyl coenzyme A (acetyl-CoA) as acetyl group donor. The liver contains the highest activity of this enzyme in most mammalian species. In humans, the extent of acetylation of certain drugs, including the antibacterial agent sulphamethazine, is genetically determined and individuals can be classified as either rapid or slow acetylators using these compounds. Polymorphic acetylation is often clinically important with respect to both drug therapy and as a predisposing host factor for a variety of clinical conditions [1].

The relationship between acetylator phenotype and diabetes remains unclear. Although conflicting results have been reported [2–7], overall analysis of the available data suggests that the frequency of rapid acetylators in diabetic patients is significantly increased [8]. However, Shenfield *et al.* [3] have suggested that the increased proportion of rapid acetylators amongst diabetic patients may not be genetically determined but is a biochemical artefact due to raised blood levels of acetyl-CoA in these subjects, a consequence of their hyperglycaemic condition. Recent preliminary results on the effect of added acetyl-CoA on the *in vitro* *N*-acetyltransferase activity of whole blood suggest that the endogenous concentration of acetyl-CoA is higher in samples from diabetic subjects [9]. However, a study with non-diabetic subjects given an oral glucose load reported that although this treatment induced minor increases in sulphamethazine clearance, the acetylator status of the volunteers was not altered [10].

In contrast to the above human studies, investigations with rats have demonstrated decreased *in vivo* acetylation capacities after induction of diabetes with either alloxan [11] or streptozotocin [12, 13]. Despite the common use of these β-cell toxins to produce animal models of diabetes, a major disadvantage of their use is the possibility of extrapancreatic toxicity. For example, the major organs responsible for metabolism and excretion of the drugs studied above are the liver and the kidney respectively and any damage to these tissues could independently alter the observed metabolic profile. The above animal studies are therefore potentially complicated by effects caused by the diabetogenic agent and which are unrelated to the induced hyperglycaemic condition. Clearly, the use of an animal which develops diabetes spontaneously would eliminate these problems. The spontaneously diabetic, insulin-dependent Bio-breeding (BB) Wistar rat is one of the best animal models of human Type 1 diabetes currently available. Particularly valuable features of this animal for use in research into insulin-dependent diabetes include a genetic predisposition to this metabolic disorder, a long pre-diabetic period and the occurrence of diabetic complications [14].

In this study, the *in vivo* acetylation capacity of adult diabetic and non-diabetic BB/Edinburgh (BB/E) rats were compared to determine the effect of diabetes on this metabolic pathway.

MATERIALS AND METHODS

Materials. Sodium sulphamethazine and sulphapyridine were supplied by the Sigma Chemical Co. (Poole, U.K.). Sulphamethazine and acetic anhydride were obtained from the Aldrich Chemical Co. (Gillingham, U.K.). Potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from the BDH Chemical Co. Ltd (Poole, U.K.). HPLC grade acetonitrile was supplied by May and Baker (Dagenham, U.K.). All other reagents were of the highest grade commercially available. Acetylsulphamethazine was synthesized from sulphamethazine by heating with acetic

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anhydride and was recrystallised from aqueous ethanol (m.p. 254–255°). The structure of the product was confirmed by mass spectrometry and its purity was 100% as judged by HPLC analysis.

Animals. The animals used in this study were from the Edinburgh colony of BB rats (BB/E), the nucleus of which was kindly donated in 1982 by Dr P. Thibert from the colony maintained at the Animal Resources Division of Canada, Ottawa. The BB/E colony consists of two sublines of animals created by selective breeding. The incidence of insulin-dependent diabetes in the diabetes-prone BB/E line (predominantly maintained by crossing diabetic male and non-diabetic female siblings) is 55–70%. The mean (±SD) age at onset of diabetes is 96 ± 18 days. The diabetes-resistant BB/E subline has an incidence of diabetes of less than 1%. Diabetic animals were treated daily with a single subcutaneous injection of insulin (Ultratard Bovine insulin, Novo, Denmark). The dose of insulin was adjusted individually on the basis of daily measurements of body weight and glycosuria. The rats were maintained at 20° on 12 hr light/dark cycles and were fed SDS rat and mouse No. 1 expanded feed (Special Diet Services, Witham, U.K.) and water *ad lib.*

Administration of sulphamethazine. Blood samples were obtained from the tail vein of the rats approximately 24 hr before administration of sulphamethazine. These samples were collected between 10 and 11 a.m. and were assayed for glycosylated haemoglobin and plasma glucose. The animals were placed in metabolic cages to acclimatise for 17 hr before administration of sulphamethazine. The sodium salt of sulphamethazine was used in these studies because of its increased solubility in aqueous solution relative to the free compound. Sodium sulphamethazine (dissolved in isotonic saline) was administered orally (via oral–gastric tube) at a dose of 40 mg/kg and in an amount of 1.0 mL/kg body weight. The rats had free access to food and water throughout the experiment. Urine samples were collected between the following time intervals; 0–4, 4–8, 8–12, 12–24 and 24–48 hr post-administration. The volumes of these samples were measured and they were then stored at −20° prior to analysis.

HPLC analysis. Urine samples were centrifuged at 400 *g* for 10 min to deposit any particulate matter. Samples collected up to 12 hr post-administration were diluted 50-fold with HPLC mobile phase before reverse phase HPLC analysis. Samples collected between 12–24 hr and 24–48 hr post-administration were diluted 20- and 10-fold respectively with HPLC mobile phase. In each case, sulphapyridine (final concentration = 25 μg/mL) was added as internal standard prior to dilution. Standard solutions of sulphamethazine and acetylsulphamethazine (0–100 μg/mL in HPLC mobile phase) containing sulphapyridine (25 μg/mL) were used to construct calibration lines. The HPLC system used to determine sulphamethazine and acetylsulphamethazine was as previously reported [15].

Analysis of urinary glucose, ketones, protein and pH. A Clinitek 2000 urine chemistry analyser (Ames Division, Miles Laboratory Incorporated, Slough, U.K.) was used to measure the pH of urine samples and to test for the presence of glucose, ketones and protein.

Determination of glycosylated haemoglobin. The percentage of glycosylated haemoglobin in rat blood samples was determined by electro-endosmosis on agarose gels [16] using densitometric scanning (Corning Medical and Scientific Equipment, Halsted, U.K.).

Glucose analysis. A Beckman Synchron AS4 automated analyser system (Beckman-RIIC, High Wycombe, U.K.) employing a glucose oxidase method was used to determine plasma glucose concentrations.

Statistics. Means and standard errors (SEM) were calculated for all data. A two tailed unpaired Student's *t*-test (population variances not assumed to be equal) was used to determine if differences between mean values were significant. The level of significance was set at $P < 0.05$.

RESULTS

Effect of diabetes on body weight, urinary output, plasma glucose and glycosylated haemoglobin

Two months duration of diabetes produced a significant decrease in body weight of the male rats although the difference in female animals was not significant (Table 1). This result may be due to administration of insufficient insulin since both male and female animals were treated with identical mean daily doses of insulin despite the significant differences in body weight of the two groups. In both the diabetic and non-diabetic groups, the mean body weight of the male rats was significantly higher than that of the female animals ($P < 0.001$). The values of all the other parameters studied were not significantly different between male and female animals within the diabetic and non-diabetic groups. Non-diabetic rats had a mean plasma glucose (non-fasting) level of 6.4 mmol/L whereas the diabetic animals exhibited significant hyperglycaemia (2- to 3-fold higher). Although the values in the latter group showed considerable variation, the differences in plasma glucose between the two groups were significantly different. Consistent with this, the mean glycosylated haemoglobin value of the diabetic rats was also significantly higher than that for the control group, suggesting long-term hyperglycaemia of the diabetic animals despite daily treatment with a long-acting insulin preparation. The daily urine production of the diabetic rats was approximately twice that of the control animals (Table 1).

Effect of diabetes on urinary levels of glucose, ketones, protein and pH

In samples obtained from the non-diabetic animals, urinary glucose levels were generally undetectable although a concentration of 5.5 mmol/L was detected in 11 of the 135 samples analysed. Table 2 illustrates the significant glycosuria observed in the diabetic animals. Seventeen of the 24 diabetic rats studied exhibited urinary glucose levels exceeding 55 mmol/L on at least one occasion during the study. There was no significant difference in urinary ketone levels between the diabetic and non-diabetic animals and the values detected never exceeded 1.5 mmol/L.

Table 1. Characteristics of diabetic and non-diabetic BB/E Wistar rats

Parameters	Non-diabetic Male (N = 12)	Non-diabetic Female (N = 12)	Diabetic Male (N = 14)	Diabetic Female (N = 10)
Age (days)	183 ± 14	174 ± 4	177 ± 4	170 ± 3
Duration of diabetes (days)	—	—	62 ± 4	67 ± 3
Daily insulin dose (units)	—	—	2.7 ± 0.1	2.7 ± 0.1
Body weight (g)	390 ± 15	244 ± 6	329 ± 8§	254 ± 7
Plasma glucose (mmol/L)*	6.4 ± 0.3	6.4 ± 0.3	12.2 ± 2.4‡	18.5 ± 3.4§
HbA1 (%)	4.8 ± 0.3	4.7 ± 0.2	7.7 ± 0.5¶	7.4 ± 0.6¶
Urine output (mL/24 hr)†	5.6 ± 0.5	6.2 ± 1.1	14.4 ± 2.8§	10.8 ± 1.5

Values represent mean ± SEM.
* Non fasting, †calculated as mean value obtained during two day period of study.
The values shown were significantly different from non-diabetic animals.
‡ $P < 0.05$.
§ $P < 0.01$.
¶ $P < 0.001$.

Table 2. Glucose concentration of urine samples from non-diabetic and diabetic BB/E rats

Animals (number)	Total No. of samples	Urinary glucose concentration (mmol/L) −ve	5.5	14	28	>=55
Non diabetic rats (N = 24)	135	92	8	—	—	—
Diabetic rats (N = 24)	131	26	19	13	16	26

The values shown represent the percentage of samples at each glucose concentration.

There was also no significant difference in the amounts of protein excreted renally by diabetic and non-diabetic rats although approximately 60% of samples from both groups had protein concentrations in the range 0.3–1 g/L. These values compare well to those reported previously in a study of glomerulopathy in the spontaneously diabetic BB rat [17]. The mean (±SEM) pH of urine samples from diabetic and non-diabetic rats were 7.8 (±0.1) and 7.6 (±0.1) respectively. These values were not significantly different ($P > 0.1$).

The effect of diabetes on the in vivo *acetylation of sulphamethazine*

HPLC analysis of urine samples collected overnight prior to sulphamethazine administration demonstrated the absence of any peaks at the retention times of sulphamethazine, acetyl-sulphamethazine and sulphapyridine. The *in vivo* acetylation of sulphamethazine by diabetic and non-diabetic BB/E Wistar rats is shown in Fig. 1. At each of the time intervals after sulphamethazine administration, the percentage acetylation is significantly higher for the diabetic animals. The acetylation capacity of male rats was significantly higher ($P < 0.001$) than female animals in each group.

Figure 2 illustrates the cumulative percentage of the administered dose recovered as sulphonamide (i.e. sulphamethazine and acetylsulphamethazine) during the 48 hr study period. The percentage of the original dose recovered during each of the time intervals studied was significantly higher ($P < 0.001$) for the female animals. There was no significant difference in the recovery of the dose between diabetic and non-diabetic rats.

DISCUSSION

This is the first reported study on the *in vivo* acetylation capacity of a spontaneously diabetic animal model. In contrast to the previous studies using chemically-induced diabetic male rats [11–13], the current study demonstrates that spontaneously diabetic male and female rats have a significantly higher acetylation capacity than non-diabetic animals. One factor which may be relevant to the conflicting results obtained using these two animal models is that the degree of metabolic disturbance in these chemically-induced diabetic animals was much greater since they were not treated with insulin. For example, the spontaneously diabetic BB/E rats used in the current study exhibited significantly less hyperglycaemia, glycosuria, hyperphagia and polyuria than that reported previously in a similar study using streptozotocin-treated

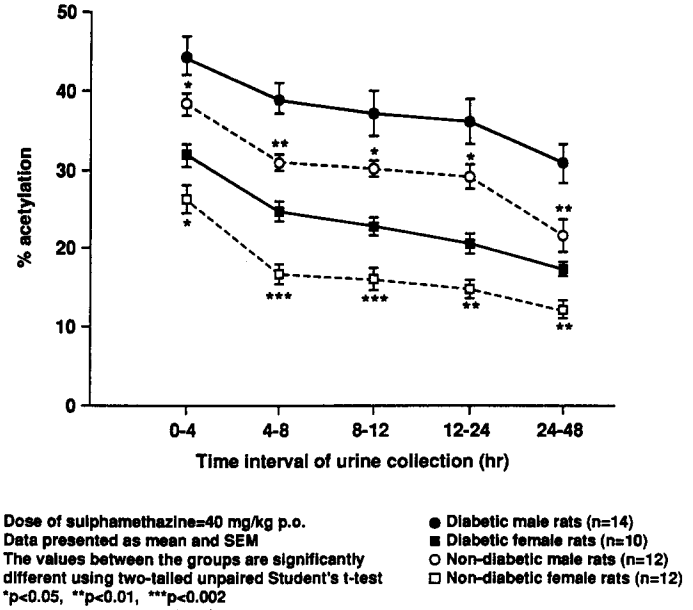

Fig. 1. *In vivo* acetylation of sulphamethazine by diabetic and non-diabetic BB/E Wistar rats.

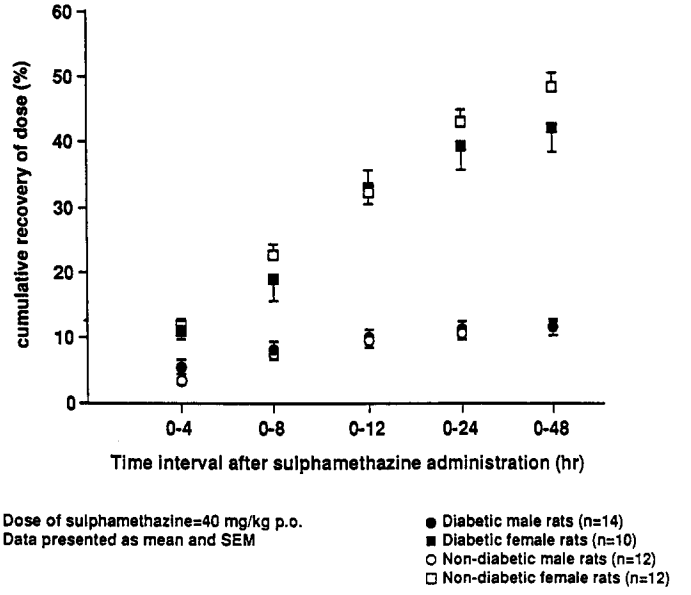

Fig. 2. Cumulative recovery of total sulphamethazine (i.e. sulphamethazine and acetylsulphamethazine) after administration to diabetic and non-diabetic BB/E Wistar rats.

animals [13]. Although the animals used in the current study were not normoglycaemic, the degree of glycaemic control observed is more comparable to that observed in human insulin-dependent diabetic patients than the severe hyperglycaemia reported in the previous studies using untreated chemically-induced diabetic rats [11–13]. The reduced metabolic disturbance observed in the current study is due to insulin treatment of the animals which was not performed in the previous studies. It is therefore possible that administration of insulin to chemically-induced diabetic rats would significantly change the *in vivo* acetylation capacity. An alternative explanation may be that treatment with streptozotocin or alloxan has effects which are additional to the induced hyperglycaemic condition and which alter the elimination of drugs metabolised by acetylation. In either case, these studies imply that the results of metabolic studies obtained using diabetogenic agents require cautious interpretation

and it may be preferable to investigate spontaneously diabetic animals if possible. However, the difficulty in animal care and breeding, the increased susceptibility to infection and the need to create, by long-term selective breeding, a subline colony of control non-diabetic animals are major disadvantages to the use of such animals [14] and probably account for the widespread use of chemically-induced diabetic animal models. However, it is apparent that the different effects of spontaneous and chemically-induced diabetes on drug metabolism will also depend on the metabolic pathways under investigation. For example, no significant quantitative or qualitative difference in the *in vitro* activities of several hydroxylase enzymes was observed in a study on the effect of diabetes on hepatic microsomal metabolism by streptozotocin-induced diabetic and spontaneously diabetic rats [18].

The metabolism of sulphamethazine by human subjects does not exhibit a significant difference between males and females [19, 20]. The previous studies on the effect of chemically-induced diabetes on drug acetylation only investigated male rats and therefore this study is the first to document a sex difference in the *in vivo* acetylation capacity of diabetic animals. A significant difference in the acetylation capacity of male and female non-diabetic rats has previously been reported [21, 22]. However, the difference observed in the current study (i.e. male rats having a higher acetylation capacity than females) is opposite to that reported previously. The reason for this discrepancy is unknown. The dose and route of sulphamethazine administration were identical in all these studies and the principal difference seems to be the strain of rats investigated. Zidek and Janku [23] suggested that the differences they observed in their previous investigations were oestrogen-dependent since administration of oestrogen either prenatally or to mature animals increased the *in vivo* acetylation of sulphamethazine by male rats. Liver *N*-acetyltransferase preparations from female rats also acetylate significantly more sulphamethazine *in vitro* than similar preparations from male animals. In contrast to the *in vivo* results, castration or oestrogen-treatment had no effect on the *in vitro* liver *N*-acetyltransferase activity of male rats [23]. As a result of these studies, it was suggested that the differences in acetylation capacity *in vivo* reflect different patterns of distribution in male and female animals [21]. This hypothesis is supported by the results of Zalalian *et al.* [24] who reported that female rats had higher ^{14}C residues in blood and selected tissues (liver, kidney, muscle and adipose tissue) after oral administration of radiolabelled sulphamethazine than male animals.

The mean recovery of the administered dose in urine samples from male animals (11.5%) in the current study was similar to the value previously reported (13.3%) using streptozotocin-induced diabetic rats [13]. An important consideration when calculating the fraction of the original dose recovered is the contribution of other metabolic pathways. The existence of other metabolites of sulphamethazine (e.g. glucuronide, sulphate and glucose conjugates) has been reported for rats both *in vivo* [24] and in studies performed *in vitro* using isolated rat hepatocytes [25] and rat whole blood [13]. No information is currently available on the effect of diabetes on the activities of the enzymes producing these additional metabolites and it is possible that diabetogenesis may change the percentages of the parent drug metabolised by the different pathways. In humans, sulphamethazine is metabolised exclusively by acetylation [26] and in this respect, the rat is not an ideal animal model. The significantly higher recovery of the original dose in samples from the female animals may indicate higher absorption since a previous study reported that female rats consistently absorbed and excreted a greater percentage of sulphamethazine in the urine than male animals at oral doses ranging from 0.83 to 207.7 mg/kg [24].

In conclusion, this study demonstrates that the *in vivo* acetylation capacity of spontaneously-diabetic, insulin-dependent BB/E Wistar rats is significantly higher than non-diabetic control animals. This result supports the majority of studies comparing this metabolic pathway in diabetic and non-diabetic human subjects [8]. In contrast to investigations using chemically-induced diabetic rats [11–13], none of the human studies [2–7] have reported reduced acetylation capacity of diabetic subjects. However, it must be re-emphasised that in the absence of studies involving insulin administration to these animal models of diabetes, the possibility that the observed effects on drug acetylation would be reversed by treatment with this hormone cannot be excluded. We are currently conducting a prospective study of the acetylation of sulphamethazine in a cohort of animals during the pre-diabetic period, at diabetes-onset and the subsequent stage of clinically established diabetes. Comparison of these results with a control group of age- and sex-matched non-diabetic animals will unequivocally clarify the relationship between diabetogenesis and *in vivo* acetylation capacity in this species.

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