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Antidiabetic Efficacy of BRL 49653, a Potent Orally Active Insulin Sensitizing Agent, Assessed in the C57BL/KsJ *db/db* Diabetic Mouse by Non-invasive ^{1}H NMR Studies of Urine

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Abstract

High resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopic analysis of biofluids is a recently established tool for evaluating inherited and acquired errors in metabolic control. In the present study ^{1}H NMR analysis of urine was used to monitor efficacy of BRL 49653, a potent and selective antihyperglycaemic agent, following oral administration for up to 36 weeks to the genetically diabetic C57BL/KsJ *db/db* mouse. The effects of BRL 49653 on carbohydrate and fatty acid metabolism were monitored by determination of changes in concentrations of low molecular weight urinary metabolites.

A qualitative comparison of the NMR spectra of urine from untreated diabetic mice with those of lean littermates and literature examples revealed several abnormalities, the majority of which could be explained in terms of the non-insulin dependent diabetes syndrome exhibited by these animals. Quantitatively the most prominent was the extreme glycosuria of both young (8–12 weeks; 0·9 g glucose kg^{-1} h^{-1}) and older (42 weeks; 2 g glucose kg^{-1} h^{-1}) diabetic mice. This was accompanied by the excretion of a number of unassigned sugar derivatives and by ketone bodies. Administration of BRL 49653 (3 μmol kg^{-1}) to *db/db* mice for 24 days reduced blood glucose concentrations to values comparable with non-diabetic lean littermates and reduced glycosuria by $> 90\%$. BRL 49653 significantly reduced excretion of unassigned sugars, acetate, lactate, and the ketone bodies, acetoacetate, 3-D-hydroxybutyrate and acetone.

The anti-diabetic efficacy of BRL 49653, assessed from the pattern of urinary metabolites, was maintained over a 36-week treatment period. These results demonstrate the value of ^{1}H NMR to evaluate non-invasively the efficacy of novel therapeutic agents.

Non-insulin dependent diabetes mellitus (NIDDM) is characterized by a diverse range of biochemical lesions that result in insulin resistance in peripheral tissues and impaired insulin secretion by the pancreas; this leads to glucose intolerance and hyperglycaemia (De Fronzo 1988; Reaven 1988). Current oral treatments have limited efficacy and alternative novel approaches, targeting insulin resistance, are being developed (Colca & Morton 1990). These agents, the thiazolidinedione insulin sensitizers, have been evaluated in a range of animal models of NIDDM.

The C57BL/KsJ *db/db* mouse is a genetic mutant that displays some of the metabolic abnormalities associated with human NIDDM (Shafrir 1992). The *db/db* mouse carries a single recessive mutation which is manifested in the homozygote as severe hyperglycaemia and hyperlipidaemia, associated with obesity and profound insulin resistance. Hyperglycaemia increases with age as a result of progressive pancreatic β-cell degranulation and necrosis (Like & Chick 1970). β-cell degeneration causes a transition from non-insulin dependent to insulin dependent diabetes, resulting finally in ketosis, weight loss and early death. Heterozygous (*db*/+) and homozygous (+/+) C57BL/KsJ mice are euglycaemic lean animals that are not hyperglycaemic or hyperlipidaemic. Thiazolidinedione-containing anti-hyperglycaemic agents, such as ciglitazone, pioglitazone and troglitazone, improve glycaemic control in animal models of NIDDM, including the *db/db* mouse, by enhancing hepatic and peripheral tissue sensitivity to endogenous insulin (Fujiwara et al 1988; Colca & Morton 1990), although continued development of some of these has been precluded because of adverse toxicology; predominantly reduced haematocrit and increased heart weight. A new thiazolidinedione, BRL 49653, is an effective anti-hyperglycaemic agent in animal models of NIDDM but is 100–200 times more potent than the earlier compounds and has a greatly reduced liability to adversely affect haematological parameters (Cantello et al 1994; Young et al 1995).

The importance of biofluid analysis in clinical chemistry and toxicology is well established. Analysis of urine, for example, provides a non-invasive means of assessing the biochemical status of the organism together with the functional capacity of the kidney. High resolution ^{1}H NMR analysis of biofluids is a recently established tool for evaluating inherited and acquired errors in metabolic control and organ function (Iles et al 1985; Nicholson & Wilson 1989). For example, the changes in ketone bodies, glucose, alanine, valine and lipoprotein concentrations in the plasma of insulin-dependent diabetic and hyperlipidaemic patients in response to therapy have been successfully monitored using ^{1}H NMR (Nicholson et al 1983). ^{1}H NMR spectroscopy has also been used successfully to diagnose inborn errors of metabolism, including propionic acidaemia, methyl malonic aciduria, branched chain ketoaciduria, isovaleric acidaemia and glutaric aciduria type I (Iles et al 1985). Its main value is as an investigative technique, cap-

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able of revealing unexpected changes in metabolite profile. This is possible because all metabolites which are present in adequate concentrations (> 50 μM, using 400 MHz detection) and which possess non-labile protons, are easily observed in the ^{1}H NMR spectrum, providing that their NMR signals do not overlap with other metabolite resonances.

In the present report we describe the use of ^{1}H NMR spectroscopy to quantify the effect of BRL 49653 on low molecular weight urinary metabolites in C57Bl/KsJ diabetic mice and lean littermates.

Materials and Methods

Animal dosing and urine collection

Female C57BL/KsJ *db/db* diabetic mice and non-diabetic (+/?) lean littermates were obtained at 4–5 weeks of age from Jackson Laboratories, Bar Harbor, USA. Mice were housed in groups of 5 (diabetics) or 7 (leans) and maintained at 26 ± 2°C on a 12 h light/12 h dark light-cycle (lights on at 06:00 h). Mice were provided with powdered RM3 diet (SDS, Special Diet Services, UK) or liquid diet (Bioserv 3, Frenchtown, USA) with free access to water. Studies began when mice were 5–6 weeks old (Study 1) or 8–9 weeks of age (Study 2). BRL 49653 ((±)-5-[[4-[2-methyl-2(pyridylamino)ethoxy]phenyl] methyl) 2,4-thiazolidinedione) was incorporated into the diet at 30 μmol (kg diet)$^{-1}$. From measurements of food intake this gives a daily dose of about 3 μmol kg^{-1} body weight.

Study 1. Diabetic mice were fed powdered RM3 diet or diet containing BRL 49653. 15 mice per group were used. After 36 weeks of compound administration 5 animals from each group were housed in a metabolism cage for 7 h and urine was collected over cardice. The volumes and pHs were determined and samples re-frozen to −70°C until NMR analysis.

Study 2. Diabetic mice and lean littermates were fed liquid diet or diet containing BRL 49653. A liquid diet formulation was used to encourage micturition from the lean littermates. Urine was collected from diabetic and lean mice before and after 24–25 days of treatment with BRL 49653. Animals from each group were housed in metabolism cages for 24 h and urine was collected over cardice into collection vials containing sodium azide. After thawing and centrifugation, the urinary volumes were measured and samples re-frozen to −70°C until NMR analysis. Urinary pH was determined at the time of NMR analysis.

^{1}H NMR analysis

A known weight of NMR standard, trimethylsilylpropionic acid-d4 (TSP), was dissolved in D_2O and 50 μL of this solution was then added to 450 μL of urine in an NMR tube. All NMR spectra were collected at 300 K on either a Bruker AMX 400 (Study 1) or AMX 500 (Study 2) NMR spectrometer using standard software. The water resonance was eliminated by gated decoupling during the relaxation delay. Metabolite identification was made with reference to literature data and by spiking authentic standards into the sample. The conditions used for NMR analysis are listed in Table 1.

All metabolites (except for glucose) were quantified using their NMR resonance peak heights in the 1-pulse, presaturation spectra. The peak heights were compared with that of the internal standard and metabolite excretion rates were calculated in μg kg^{-1} body weight h^{-1} using the following equation:

$$\frac{Py \times M(TSP) \times N(TSP) \times V_2 \times RMM(y) \times 10^3}{Ny \times P(TSP) \times V_1 \times W \times h} \quad (1)$$

where TSP = internal standard, y = metabolite of interest, P = peak height in cm, M = concentration in mM, N = number of protons in the NMR resonance, V_1 = volume of urine in the NMR tube, V_2 = total volume of urine excreted, RMM = molecular weight, W = total weight of mice in the metabolism cage, h = number of hours for which urine was collected.

Table 1. Experimental conditions for 1-Pulse NMR experiments.

	Study 1	Study 2
Spectrometer	Bruker AMX 400	Bruker AMX 500
Frequency	400 MHz	500 MHz
Probe	BB-VSP	BB-VSP
Spectral width	6849·49	6024·10
Transients	600	256
Steady-state transients	4	4
Data points in FID	32768	32768
Data points in spectrum	32768	32768
Relaxation delay	3	3
Line broadening	0·3	0·5
Presaturation pulse	3s	3s

Due to partial saturation of the glucose anomeric signals during solvent suppression, the glucose quantitative measurements at 400 MHz in Study 1 were recorded using Carr–Purcell–Meiboom–Gill (CPMG) spin-echo, rather than 1-pulse, NMR techniques.

50 μL 5 M guanidinium chloride in D_2O, plus 50 μL TSP in D_2O were added to a known volume of urine and made up to a final volume of 500 μL in an NMR tube. The sample pH was adjusted to pH* = 8·4 using DCl and NaOD [to produce intermolecular proton exchange at an intermediate rate and hence reduce the water T_2 relaxation time before spectral acquisition (Connor et al 1987)].

The effect of the CPMG pulse sequence on metabolite signal attenuation relative to that of TSP was investigated using a range of total echo time, τ, values (54, 66, 78, 96 and 120 ms, where the interpulse delay was 1·5 ms). It was found that on spiking a known amount of glucose standard into the sample, the percentage increase in peak height relative to the peak height of TSP did not vary with t. Glucose concentration was therefore estimated by recording CPMG spectra before and after the addition of glucose into the sample, using a total t of 120 ms, which allowed almost complete suppression of the water resonance.

Quantitative NMR data for the urinary metabolites of lean and diabetic mice from Study 2 were acquired at 500 MHz using a lower presaturation power than for Study 1, allowing more accurate quantitation of the glucose in the 1-pulse spectra.

Assignment of β-glucosan

In an attempt to identify the major unassigned resonances *observed* between 3·5 and 5·5 ppm, additional experiments

were carried out on a concentrated sample of 10 mL of urine collected from control lean mice which was freeze-dried and reconstituted in 0·5 mL of D_2O. 1D and 2D NMR experiments were carried out at 400 MHz using water suppression by gated decoupling during the relaxation delay.

Enzymic determination of glucose

Urinary and blood glucose concentrations (determined in 10 μL blood samples taken from the cut tip of the tail of conscious mice) were measured spectrophotometrically with hexokinase/glucose-6-phosphate dehydrogenase (Cantello et al 1994).

Materials

Deuterated solvents were obtained from Fluorochem, TSP standard from Wilmad Glass Co. Inc., USA and guanidinium chloride from BDH. Metabolite standards were obtained from Sigma (glucose-6-phosphate, hippuric acid, creatine, fructose, succinate, taurine, 2-oxoglutarate, 3-D-hydroxybutyrate and acetoacetate), Aldrich (creatinine, DMA, DMG, TMA, and TMAO hydrochlorides), BDH (glucose, sodium formate), and Fisons (alanine, sodium acetate, acetone).

Results

Profile of urinary metabolites in control diabetic and non-diabetic lean mice

The spectra and quantitative NMR data of urine samples from young diabetic mice of 12–13 weeks of age were markedly different from the corresponding spectra for lean litter mates (Fig. 1; Table 2). The most obvious difference was the pronounced increase in urinary glucose concentration, which when corrected for the increased urinary production rate, approximated to a 1000-fold greater excretion of glucose by diabetic mice (20 g kg^{-1} day^{-1}). Measurements of glucose excretion by NMR agreed well with those assessed by enzymatic assay (Table 3). Glycosuria of diabetic mice correlated with a significant hyperglycaemia (22·4 mmol L^{-1} compared to 7·8 mmol L^{-1} in non-diabetic mice).

A number of unassigned resonances between 5 and 5·6 ppm were also detected in diabetic mouse urine spectra, the chemical shift and coupling constant data for which suggested that they corresponded to other sugar derivatives. In addition, levels of acetate, lactate and citric acid cycle intermediates were significantly increased in diabetic animals. Ketone bodies (acetoacetate, 3-hydroxybutyrate and acetone) were not detected in either the young lean or diabetic mice.

The most prominent metabolite in lean mouse urine corresponded to a novel set of resonances between 3·5 and 5·5 ppm not previously observed in urine spectra. Initial experiments using 2D COSY-45 NMR to identify these unassigned resonances were unsuccessful due to peak overlap with the water and glucose resonances. However using a combination of Hahn spin echo, 1D selective TOCSY, 1D ^{13}C DEPT135 and 2D carbon-proton HMQC, HMBC and HMQC-TOCSY NMR procedures it was possible to assign the major unidentified resonances to a novel metabolite, β-glucosan. In particular, the 1D-selective TOCSY confirmed that the resonances at 4·11, ca 3·78 and 4·65 ppm belonged to the same spin system and therefore the same molecule (Table 4, Fig. 2). The HMQC-

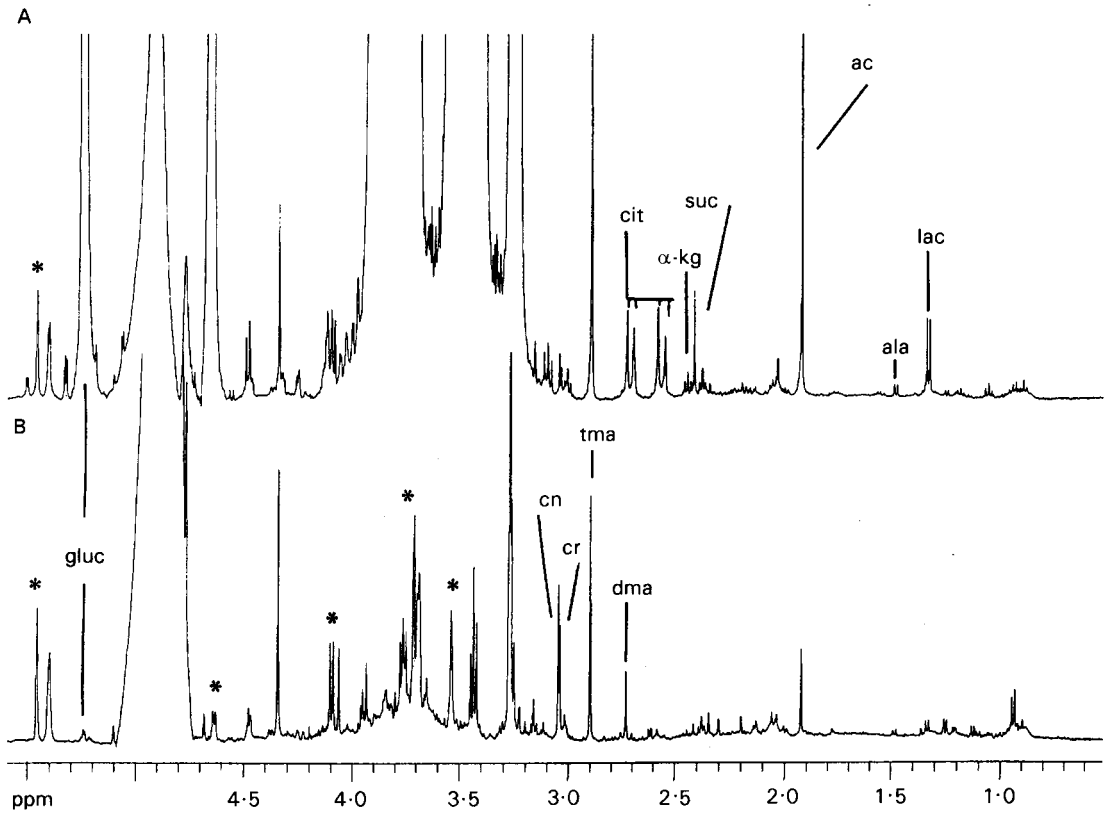

FIG. 1. Partial 500 MHz ^1H NMR spectra of urine collected in Study 2 from control diabetic *db/db* mice (A) and control + /? lean litter mates (B). *Novel resonances corresponding to β-glucosan; ac = acetate; ala = alanine; αkg = α-ketoglutarate; cn = creatinine; cr = creatine; cit = citrate; dma = dimethylamine; gluc = glucose; suc = succinate; tma = trimethylamine.

Table 2. Effect of short-term (24-day) BRL 49653 treatment on urinary metabolite content of *db/db* and +/? non-diabetic mice, quantitated by ^{1}H NMR.

Metabolite	δ_H (ppm)	Metabolite (μg kg^{-1} h^{-1})			
		Diabetic control	Diabetic + BRL 49653	Non-diabetic control	Non-diabetic + BRL 49653
Acetate	1·93	1000	717	329	208
Acetoacetate	2·24	ND	ND	ND	ND
acetone	2·29	ND	ND	ND	ND
3-Hydroxybutyrate	1·21	ND	ND	ND	ND
TMA	2·90	605	442	171	150
Creatine	3·05	133	88	492	505
Creatinine	3·06	225	225	667	659
Glucose α	5·24	356×10^3	20×10^3	375	334
Glucose β	4·65	557×10^3	39×10^3	571	529
Lactate	1·34	492	279	100	75
Alanine	1·54	92	42	42	33
Succinate	2·42	359	288	63	67
2-Oxoglutarate	2·45	292	ND	ND	ND
Citrate	2·55	1084	152	ND	ND
DMA	2·74	54	63	63	58
DMG	2·94	ND	ND	ND	ND
Propionate	1·04	96	79	ND	ND
TMAO	3·30	(1)	(1)	776	792
Taurine	3·42	(1)	(1)	1747	2319
β-Glucosan	4·11	2961	2836	3627	3252

Urine was collected from mice over a 24-h period after 24 days treatment with BRL 49653 (30 μmol (kg of diet)$^{-1}$). Data are presented as cumulative values of 15 mice per treatment group. ND = not detected in NMR spectra. (1) not possible to quantify due to peak overlap with glucose. TMA = trimethylamine; glucose α = α anomer of glucose; glucose β = β anomer of glucose; DMA = dimethylamine; DMG dimethylglycine; TMAO = trimethylamine-*N*-oxide.

Table 3. Effect of BRL 49653 on blood glucose concentration and urinary glucose excretion in genetically diabetic mice and lean littermates.

Group	Blood glucose concn (mmol L^{-1})		Urinary glucose (α & β anomers) concn (mmol L^{-1})			
	Pre-dose	Post-dose	Pre-dose		Post-dose	
			NMR	Enzymic	NMR	Enzymic
Lean control	5·7 ± 0·8	7·8 ± 0·8	0·2	0·6	0·5	0·6
Lean + BRL 49653	5·7 ± 0·7	7·8 ± 1·0	0·5	0·6	0·5	0·7
Diabetic control	14·2 ± 3·8	23·4 ± 6·7	195	244	511	578
Diabetic + BRL 49653	13·8 ± 3·0	10·0 ± 4·4***	193	198	50	64

Blood glucose concentrations of fed mice were determined before (mice aged 8–9 weeks; pre-dose) and after 24 days of treatment with BRL 49653 (30 μmol (kg of diet)$^{-1}$; post-dose). Results are means ± s.d. (n = 15). ***$P < 0.001$ vs untreated diabetic mice by Student's *t* test. Glucose was determined enzymically or by NMR in urine collected over the final 24 h of treatment with BRL 49653. Urinary data are cumulative data of 15 mice per group.

TOCSY experiment (Fig. 3) showed, via correlations from protons detected in the selective TOCSY experiment, that the unknown metabolite contained at least 6 protonated carbons, each attached to oxygens, one of which was characteristic of a sugar anomeric centre ($\delta_C = 104{\cdot}2$ ppm, $\delta_H = 5{\cdot}49$ ppm), and another ($\delta_C = 67{\cdot}9$ ppm, $\delta_H = 4{\cdot}13$, 3·78 ppm) which was assigned on the basis of DEPT135 data to a CH_2 group. Subsequent comparison with literature data (Pouchert & Behnke 1993) indicated that the novel metabolite corresponded to β-glucosan (Fig. 4, Table 4) which was later confirmed by spiking authentic compound into the sample.

Using the resonance at 4·11 ppm it was possible to detect the presence of β-glucosan in all spectra of urine from diabetic and lean animals that were fed liquid diet (Study 2) but not in the urine collected from older mice fed powdered RM3 diet (Study 1).

Age-related changes in urinary metabolite profile of diabetic mice

The profile of urinary metabolites from older diabetic mice (approx 36 weeks; study 1) was qualitatively similar to that of younger mice (12–13 weeks; study 2), although quantitatively the glycosuria was more marked (Tables 2 and 5) and the concentrations of unknown sugar metabolites were also increased (Fig. 5, top trace). In addition, resonances from the ketone bodies, acetoacetate, acetone and 3-hydroxybutyrate, were observed in the older mice.

BRL 49653-induced changes in urinary metabolite excretion

The effect of BRL 49653 on control of glycaemia and pattern of urinary metabolite excretion in diabetic mice was determined initially after administration of the compound in the diet for a period of 24 days (Table 2). NMR data indicated that

Table 4. NMR data for β-glucosan in urine.

Atom	$\delta_C$ppm*†	$\delta_H$ppm*	Multiplicity	^nJ values (Hz)	HMBC correlations	HMQC-TOCSY
1	104·2	5·49	poorly resolved triplet	$^4J_{H1,H3}=^3J_{H1,H2}=1.6$	H6, H3	H1, H2, H3/4
2	72·7	3·57	poorly resolved quartet	$^4J_{H2,H4}=^3J_{H1,H2}=^3J_{H3,H2}=1.6$	H1, H4	H1, H2, H3/4
3	75·3	3·73	poorly resolved multiplet		H1, H5, H4 H2	H1, H2, H5, H3/4
4	73·4	3·72	poorly resolved multiplet		H5, H6, H3	H6
5	79·1	4·65	doublet	$^3J_{H5,H6}=5.9$	H1, H6	H6, H3/H4, H5
6	67·9	4·13	doublet	$^2J=7.6$	H1	H5, H6
		3·78	doublet of doublets	$^3J_{H5,H6}=5.9$, $^2J=7.6$		

*Referenced to δ TSP = 0 ppm. †^{13}C Chemical shift literature values for β glucosan in D_2O = 104·2, 79·1, 75·3, 73·6, 73·0, 68·0 (Pouchert & Behnke 1993).

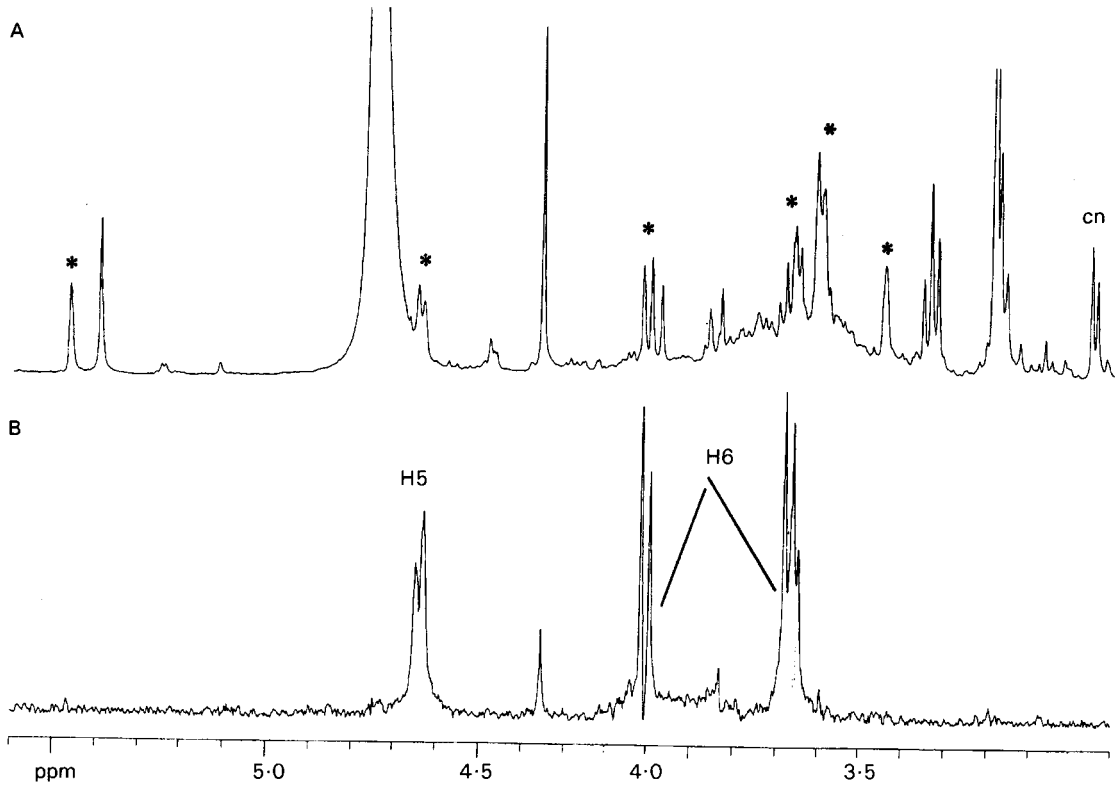

FIG. 2. Partial 400 MHz ^{1}H NMR spectra of freeze dried control urine collected from +/? lean litter mates in Study 2 (A) and a selective ^{1}H 1D TOCSY of the same sample (B) irradiating the doublet at 4·11 ppm. The 1D TOCSY data was acquired using 96 transients, 4 steady-state transients and a 1K selective guassian-shaped 25 ms 90 pulse with 6% truncation and 61 dB attenuation. The water signal was suppressed using presaturation in the relaxation delay and a spin lock of 67 ms was achieved using an MLEV17 sequence. The experiment confirmed that the peaks labelled H5 and H6 were part of the same spin system. (Abbreviations as for Fig. 1.)

urinary excretion of glucose by control diabetic mice increased about 2·5-fold during the study. This correlated well with the significant increase in blood glucose concentration from 14·2 to 22·4 mmol L^{-1} (Table 3). Treatment of diabetic mice with BRL 49653 reduced blood glucose concentrations to values close to those determined in non-diabetic mice and reduced urinary glucose excretion by about 90%, although in this study urinary glucose of treated diabetic mice was still significantly greater than in non-diabetic littermates. BRL 49653 treatment did not produce any change in excretion of lactate, acetate or of any unassigned resonances in diabetic mice nor did the compound influence blood glucose or urinary metabolite profiles of non-diabetic mice.

The ability of BRL 49653 to maintain glycaemic and metabolic control over an extended period was determined in a parallel experiment in which BRL 49653 was administered for

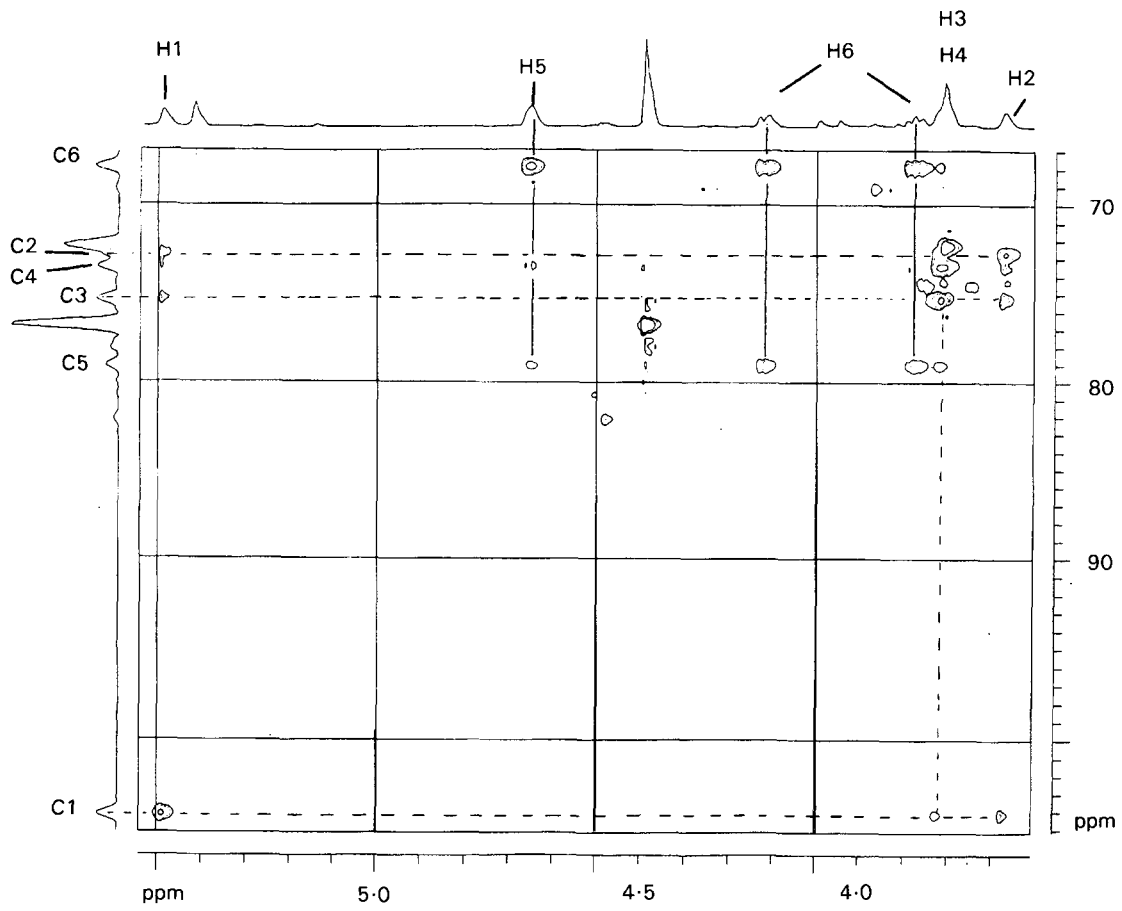

FIG. 3. A partial 400 MHz ^{1}H, ^{13}C 2D HMQC-TOCSY NMR spectrum of control urine collected from + /? lean litter mates in Study 2. Data was acquired using 4K data points, 512 t1 increments, 88 transients, 4 steady-state transients, a 67 ms spin lock and a 3·7 ms delay to allow evolution of $^{1}J_{CH}$ coupling corresponding to 140 Hz. ^{13}C decoupling was achieved using a GARP sequence during data acquisition. Heteronuclear couplings from H5 and H6 observed in the HMQC spectrum (data not shown) and HMQC-TOCSY data confirmed the assignments of their attached carbons and C4, whilst the additional coupling observed from H4 to C5 led to the assignments of C1, H1, H2 and hence C2 and C3 (Table 4, Fig. 4).

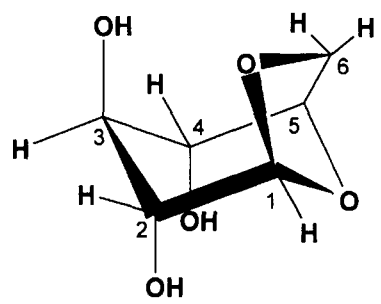

FIG. 4. Structure of β-glucosan.

36 weeks (Table 5, Fig. 5). Treatment with BRL 49653 resulted in a greater than 1000-fold reduction in the urinary excretion of glucose anomers relative to the untreated controls. There was also a substantial reduction in the amount of unassigned putative sugars in the urine, with the exception of the metabolites resonating at 5·46 and 4·67 ppm (observed in CPMG spectra and tentatively assigned to maltose on the basis of chemical shift) which had comparable concentrations to glucose itself. Substantially lower urinary concentrations of acetate and lactate were observed in animals treated with BRL 49653. In addition, there were only trace amounts of ketone bodies observed in treated animals. The intensity of an unassigned triplet resonance at 2·38 ppm (which did not correspond to glutamine or glutamate) was increased in animals after long-term administration of BRL 49653.

Discussion

Use of ^{1}NMR to characterize the metabolic status of the C57BL/KsJ mouse model of NIDDM

The C57BL/KsJ *db/db* diabetic mouse displays many of the metabolic abnormalities of the human non-insulin dependent diabetic and has therefore been extensively used to assess both the pathology of disease progression and the efficacy of novel antihyperglycaemic agents. In young diabetic mice, the development of hyperglycaemia at 6–8 weeks of age is associated with insulin resistance in target tissues; plasma-insulin concentrations are increased in an attempt to mainatain normoglycaemia. As the animal ages, a progressive deterioration of pancreatic β-cell function develops, resulting eventually in hypoinsulinaemia and severe hyperglycaemia, glycosuria and ketoacidosis. Since changes in urinary concentrations of glu-

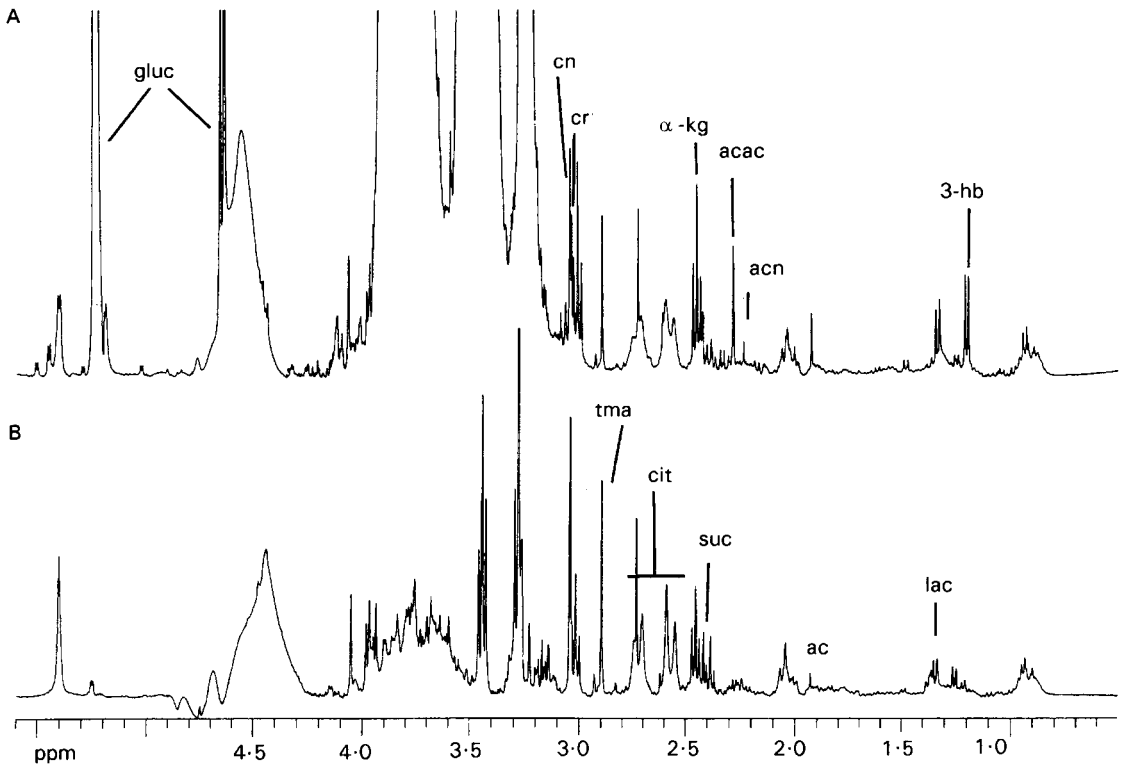

FIG. 5. Partial 400 MHz ^{1}H NMR spectra of urine collected from control diabetic *db/db* mice in Study 2 (A) and diabetic mice treated for weeks with BRL 49653 (B). 3hb = 3-D-hydroxybutyrate; acn = acetone; acac = acetoacetate.

Table 5. Effect of long-term (36-weeks) BRL 49653 treatment on urinary metabolite content of *db/db* mice, quantitated by ^{1}H NMR.

Metabolite	δ_H (ppm)	Urinary metabolite (μg kg^{-1} h^{-1})	
		Diabetic control	Diabetic + BRL 49653
Acetate	1·93	268	18
Acetoacetate	2·24	784	17
Acetone	2·29	50	ND
3-Hydroxybutyrate	1·21	1531	38
TMA	2·90	200	75
Creatine	3·05	340	299***
Creatinine	3·06	1472	470***
Glucose α*	5·24	645×10^3	791
Glucose β*	4·65	1362×10^3	634
Lactate	1·34	703	81
Alanine	1·54	132	15
Succinate	2·42	263	87
2-Oxoglutarate	2·45	5432	755
DMA	2·74	306	112
DMG	2·94	326	112
TMAO	3·29	—**	378***
Taurine	3·42	—**	909***
Hippurate	2·94	32	18
Formate	8·47	17	12

Urine was collected from mice over a 7 h period after 36 weeks of treatment with BRL 49653 (30 μmol (kg of diet)$^{-1}$). Data are presented as cumulative values of 15 mice per group. ND = not detected in NMR spectra. Each metabolite, unless stated otherwise, was quantified using its peak height relative to TSP in the presaturation 1-pulse NMR experiments. *Quantified using relative peak height to TSP in CPMG studies. **Peak overlap with glucose. ***Calculated using the increase in relative peak height produced by spiking with a known amount of metabolite standard. Abbreviations are detailed in the legend to Table 3.

cose and lipid-derived metabolites mirrors those in the circulation, non-invasive ^{1}H NMR techniques might be applicable to monitor both the progression of the diabetic state and assess the impact of therapeutic intervention.

A comparison of NMR spectra of urine from young diabetic mice and their lean littermates with literature data (Nicholson & Wilson 1989) showed that there were substantial abnormalities in the urinary metabolite profile of diabetic mice. Some of these differences, particularly the extreme glycosuria, are a direct reflection of the elevated blood glucose concentration. The presence of a large number of unassigned, putative sugar resonances between 4·5 and 5·5 ppm, which may include maltose, glucuronides or other metabolites or oligomers of glucose, may also be a result of the high concentration of circulating glucose. The origin of the β-glucosan excreted by mice fed on the liquid diet, but not powdered RM3 diet, is unclear since it is not a dietary component (Bioserv personal communication). The possibility of β-glucosan formation via direct pyrollysis of glucose in the diet can be discounted. In addition, β-glucosan excretion was not a consequence of diabetes, since it was present in the urine of both non-diabetic and diabetic mice. β-Glucosan has previously been detected in human urine using HPLC analysis (Dorland et al 1986).

The detection of ketone bodies in the urine samples from old (approx 36 weeks) but not young (2–3 months) diabetic mice or normoglycaemic lean mice is consistent with measurements of blood concentrations of ketone bodies, which increase significantly when pancreatic β-cell function declines to such an extent to permit unrestrained lipolysis from adipose tissue and therefore increased hepatic non-oxidative metabolism of fatty acids to acetoacetate, 3-hydroxybutyrate and acetone. Urinary excretion of lactate was increased in younger diabetic animals compared to lean littermates. This may be caused by a number of factors associated with the pathology of NIDDM. For instance, lactate oxidation via the TCA cycle is impaired in insulin resistant states, probably as a consequence of increased lipid oxidation. In addition, lactate excretion may be increased as a consequence of impaired renal tubular reabsorption (Holmes et al 1992). Similarly, creatinine excretion rates were greater in older diabetic mice than in younger animals, suggesting further that renal function was impaired. Morphological studies clearly demonstrate the age-dependent development of nephropathy in the *db/db* mouse (Lee & Bressler 1981).

Biofluid NMR is clearly a particularly useful tool for the simultaneous quantitation of a number of indices of metabolic control and renal function, some of which are impossible to measure non-invasively, and for assessing the time course of disease progression.

Effects of BRL 49653 on the blood and urinary metabolite profile of C57BL/KsJ db/db diabetic mice

BRL 49653 significantly reduces fasting hyperglycaemia and improves glucose tolerance after a period of repeat administration (8–21 days) in the obese mouse and Zucker fatty rat models of NIDDM (Smith et al 1993; Young et al 1995). Neither of these models, however, develops severe hyperglycaemia, glycosuria, ketoacidosis or pancreatic β-cell failure and we therefore determined the impact of BRL 49653 treatment on urinary glucose and ketone body production, surrogate markers of antihyperglycaemic activity and pancreatic β-cell reserve, respectively, in the *db/db* mouse.

Administration of BRL 49653 for 24–25 days to young diabetic mice (8 weeks of age at the start of treatment) produced significant reductions in both blood glucose concentrations and urinary glucose excretion (19-fold compared to control diabetic mice), although glycosuria was not totally abolished. Prolonged administration resulted in virtual elimination of glycosuria which is consistent with long-term maintenance of blood glucose concentrations below the renal glucose threshold and therefore within the normal range. BRL 49653 treatment also reduced concentrations of the unknown putative sugar metabolites, the anomeric protons of which resonated between 4·5 and 5·5 ppm. It is not known whether BRL 49653 has a selective action on the production or disposal, or both, of these unknown metabolites or whether their formation in control diabetic animals is merely enhanced by the prevailing hyperglycaemia.

Long-term (36 weeks) treatment of diabetic mice with BRL 49653 reduced urinary excretion of acetate and ketone bodies, suggesting that development of ketosis was prevented. This is again consistent with the conclusion that BRL 49653, by normalising glycaemia and preserving β-cell function, reduces lipolysis and hepatic ketogenesis, although direct measurements are required to confirm this. Creatinine excretion rates were significantly reduced in older diabetic mice treated with BRL 49653, suggesting that the compound also has a renal protective action. Whether this is a direct renal action of BRL 49653 or is a secondary consequence of improved glycaemic control is unclear, but BRL 49653 also prevents the development of nephropathy in the Zucker fatty rat which is not overtly hyperglycaemic (Al-Barazanji et al 1995).

In conclusion, the results presented here show that the analysis of urinary metabolite profiles by NMR spectroscopy is a useful tool for studying non-invasively metabolic abnormalities and drug efficacy in animal models of human disease states. Several abnormalities associated with NIDDM-like pathology have been identified in the urinary metabolite profile of genetically diabetic mice and, in particular, NMR analysis has provided qualitative and quantitative biochemical information on the antihyperglycaemic activity of BRL 49653.

References

Al-Barazanji, K., Buckingham, R. E., Turner, N., Clapham, J. C., Slaughter, M. (1995) BRL 49653 protects against nephropathy in obese Zucker rats: functional evidence. Diabetes 44 (Suppl. 1): 71A

Cantello, B. C. C., Cawthorne, M. A., Cottam,G. P., Duff, P. T., Haigh, D., Hindley, R. M., Lister, C. A., Smith, S. A., Thurlby, P. L. T. (1994) [[ω-Heterocyclylamino)alkoxy]benzyl]-2,4-thiazolidinediones as potent antihyperglycaemic agents. J. Med. Chem. 37: 3977–3985

Colca, J. R., Morton, D. R. (1990) Antihyperglycaemic thiazolidinediones: ciglitazone and its analogues. In: Bailey, C. J., Flatt, P. R. (eds) New Antidiabetic Drugs. Smith-Gordon, London, pp 255–261

Connor, S. C., Nicholson, J. K., Everett, J. R. (1987) Chemical exchange and paramagnetic T2 relaxation agents for water suppression in spin-echo proton NMR spectroscopy of biofluids. Anal. Chem. 59: 2885–2891

De Fronzo, R. A. (1988) The triumvirate: β-cell, muscle, liver: a collusion responsible for NIDDM. Diabetes 37: 667–687

Dorland, L., Wadman, S. K., Fabery de Jonge, H., Ketting, D. (1986) 1,6-anhydro-β-D-glucopyranose (β-glucosan), a constituent of human urine. Linica Chimica Acta 159: 11–16

Fujiwara, T., Yoshioka, S., Yoshioka, T., Ushiyama, I., Horikoshi, H. (1988) Characterisation of new oral antidiabetic agent CS-045. Studies in KK and ob/ob mice and Zucker fatty rats. Diabetes 37: 1549–1558

Holmes, E., Bonner, B. C., Sweatman, B. C., Lindon, J. C., Beddell, C. R., Rahr, E., Nicholson, J. K. (1992) Nuclear magnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury (2) chloride and 2-bromoethanamine. Mol. Pharm. 42: 922–930

Iles, R. A., Hind, A. J., Chalmers, R. A. (1985) Use of proton nuclear magnetic resonance spectroscopy in detection and study of organic acidurias. Clin. Chem. 31: 1795–1801

Lee, S. M., Bressler, R. (1981) Prevention of diabetic nephropathy by diet control in the *db/db* mouse. Diabetes 30: 106–111

Like, A. A., Chick, W. L. (1970) Studies in the diabetic mutant mouse I. Light microscopy and radioautography of pancreatic islets. Diabetologia 6: 207–215

Nicholson, J. K., Wilson, I. D. (1989) High resolution proton magnetic resonance spectroscopy of biological fluids. Progress in NMR Spectroscopy 21: 449–501

Nicholson, J. K., Buckingham, M. J., Sadler, P. J. (1983) High resolution ^{1}H NMR studies of vertebrate blood and plasma. Biochem. J. 211: 605–615

Pouchert, C. J., Behnke, J. (1993) Aldrich Library of ^{1}H and ^{13}C FT-NMR spectra. 2nd edn, Aldrich Chemical, Milwaukee, WI

Reaven, G. M. (1988) Role of insulin resistance in human disease. Diabetes 37: 1595–1607

Shafrir, E. (1992) Animal models of non-insulin dependent diabetes. Diabetes Metab. Rev. 8: 179–208

Smith, S. A., Cawthorne, M. A., Coyle, P. J., Holder, J. C., Kirkham, D. M., Lister, C. A., Murphy, G. J., Young, P. W. (1993) BRL 49653 normalises glycaemic control in Zucker *fa/fa* rats by improving hepatic and peripheral tissue sensitivity to insulin. Diabetologia 36 (Suppl. 1): A184

Young, P. W., Cawthorne, M. A., Coyle, P. J., Holder, J. C., Holman, G. D., Kozka, I. J., Kirkham, D. M., Lister, C. A., Smith, S. A. (1995). Repeat treatment of obese mice with BRL 49653, a novel and potent insulin sensitiser, enhances insulin action in white adipocytes: association with increased insulin binding and cell surface glucose transporter, (GLUT 4), as measured by photoaffinity labelling. Diabetes 44: 1087–1092