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Selective inhibition of 11β-hydroxysteroid dehydrogenase type 1 decreases blood glucose concentrations in hyperglycaemic mice

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Abstract

Aims/hypothesis. Current pharmacological treatments for Type II (non-insulin-dependent) diabetes mellitus have various limitations. New treatments are needed to reduce long-term risks for diabetic complications and mortality. We tested a new principle for lowering blood glucose. It is well known that glucocorticoids in excess cause glucose intolerance and insulin resistance. The enzymes 11β-hydroxysteroid dehydrogenase type 1 and type 2 inter-convert inactive and active glucocorticoids, thereby playing a major role in local modulation of agonist concentration and activation of corticosteroid receptors in target tissues. It has been hypothesized that selective inhibition of 11β-hydroxysteroid dehydrogenase type 1 decreases excessive hepatic glucose production in hyperglycemia and diabetes. BVT.2733 is a new, small molecule, non-steroidal, isoform-selective inhibitor of mouse 11β-hydroxysteroid dehydrogenase type 1. The aim of the present study is to test if selective inhibition of 11β-hydroxysteroid dehydrogenase type 1 lowers blood glucose concentrations in a hyperglycaemic and hyperinsulinaemic mouse model.

Methods. BVT.2733 was given to spontaneously hyperglycaemic KKA^{y} mice for 7 days using subcutaneous osmotic mini-pumps.

Results. BVT.2733 lowered hepatic PEPCK and glucose-6-phosphatase mRNA, blood glucose and serum insulin concentrations compared with vehicle treated mice. In contrast, hepatic 11β-hydroxysteroid dehydrogenase type 1 mRNA, liver function marker enzyme expression (aspartate aminotransferase, alanine aminotransferase and alkaline phosphatases), daily food intake and body weight were not altered by the treatment.

Conclusion/interpretation. These results suggest that a selective inhibitor of human 11β-hydroxysteroid dehydrogenase type 1 can become a new approach for lowering blood glucose concentrations in Type II diabetes. [Diabetologia (2002) 45:1528–1532]

Keywords Hydroxysteroid dehydrogenases, 11β-HSD1, blood glucose, gluconeogenesis, glucose-6-phosphatase, hyperglycaemia, phosphoenolpyruvate carboxykinase (GTP), pharmacology, oral pharmacotherapy, diabetes.

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Abbreviations: 11,; β ,; -HSD1, 11β -hydroxysteroid dehydrogenase type 1; ALP, alkaline phosphatases; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BVT.2733, 3-chloro-2-methyl-N-{4-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-1,3-thiazol-2-yl} benzenesulfonamide; FAM, 6-carboxyfluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; PBS, phosphate buffered saline; TAMRA, 6-carboxytetramethylrhodamine.

The liver is central in glucose homeostasis and plays a major role in the aetiology of glucose intolerance and Type II (non-insulin-dependent) diabetes mellitus. Under normal conditions gluconeogenesis, that is the synthesis of glucose from lactate, pyruvate, glycerol and glucogenic amino acids accounts for approximately 25% of endogenous hepatic glucose production, the rest being due to glycogenolysis [1]. However, in Type II diabetes as much as 90% of the hepatic glucose output can be due to accelerated gluconeogenesis [1]. Glucocorticoids are potent functional antagonists of insulin action and are essential for increased hepatic glucose output and raised blood glucose concentrations in

diabetes [2, 3, 4, 5]. Glucocorticoids are involved in the transcriptional control of several genes involved in the regulation of hepatic glucose production, including *PEPCK*, encoding the enzyme catalysing the rate-limiting step in gluconeogenesis [6].

Tissue response to glucocorticoids is regulated by the glucocorticoid receptor and intracellular synthesis of active glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1; EC 1.1.1.146). In humans, 11β-HSD1 converts cortisone into active cortisol [7, 8, 9, 10, 11]. In rodents, the inactive glucocorticoid is 11-dehydrocorticosterone and the active is corticosterone. *11β-HSD1* is widely distributed and is highly expressed in human liver and is also found in adipose tissue [10, 12] and pancreatic beta cells [13]. Another enzyme, 11β-HSD type 2 catalyses the reverse reaction and is found for example in kidney and placenta [14, 15, 16]. Inhibition of the type 2 enzyme in the kidney results in serious conditions associated with inappropriate mineralocorticoid receptor activation by glucocorticoids, like sodium retention, hypokalaemia and hypertension [10, 17].

11β-HSD1 alters glucocorticoid hormone action in target tissues for insulin action and has been suggested to play a regulatory role in glucose homeostasis. In a clinical study, a non-selective 11β-HSD1 inhibitor has been shown to enhance insulin sensitivity in healthy subjects [18]. Moreover, studies done in the *11β-HSD1* gene knock-out mice suggest that enzyme inhibition could decrease blood glucose concentrations without risk for hypoglycaemia [19]. Both fasting blood glucose concentrations and hepatic transcription of PEPCK and glucose-6-phosphatase (G6Pase) mRNA were lower in the *11β-HSD1* gene knock-out mice compared with wild-type mice [19]. In contrast, overexpression of the *11β-HSD1* gene in adipose tissue caused hyperglycaemia, glucose intolerance, hyperinsulinaemia, insulin resistance, and visceral obesity [20].

Due to lack of specific 11β-HSD1 inhibitors [21], non-selective inhibitors of both 11β-HSD enzymes type 1 and type 2, including glycyrrhizic acid, glycyrrhetinic acid and carbenoxolone, have been used in previous attempts to assess the functional role of 11β-HSD1 in glucose homeostasis [18]. BVT.2733 is a new type 1 selective inhibitor of murine 11β-HSD1 (K_i 1 μmol/l, using 11-dehydrocorticosterone as substrate), which does not inhibit mouse 11β-HSD type 2 at a concentration as high as 200 μmol/l (corresponding to a K_i>33 μmol/l, using 50 nmol/l corticosterone as substrate; R. Olsson, personal communication). Thus, the selectivity of BVT.2733 for the 11β-HSD type 1 enzyme over the type 2 enzyme is estimated to be at least 30-fold based on the K_i values. Our experiments focused on investigating the effects of inhibition of hepatic 11β-HSD1 on glycaemic control in hyperglycaemic mice.

Materials and methods

Animals. Male KKA^y mice, age 6 to 12 weeks (Clea, Tokyo, Japan), were housed individually at 22±1°C, 12:12 h light-to-dark cycle, and fed a high-fat diet (32.5 kcal% fat, D12266B; Research Diets, New Brunswick, N.J., USA) and water ad libitum at least 3 weeks before the experiments. Animals were grouped according to 4-h fasting blood glucose concentrations. The procedures involving animals were in conformity with national and international laws for the care and use of laboratory animals. The local animal ethics committee approved the experiments.

Animals were anaesthetized with enflurane (Efrane, Abbot Scandinavia, Solna, Sweden). Alzet (Alza, Palo Alto, Calif., USA) osmotic mini-pumps (model 2001; 0.2 ml, pumping rate 1.0 μl/h) were inserted subcutaneously (s.c.) on the back of the neck. In a separate experiment, hepatic 11β-HSD1 inhibition was measured after oral BVT.2733 administration.

Hepatic 11β-HSD1 activity. Snap frozen liver lobes (0.5–2 g) in liquid nitrogen, stored at –70°C, were homogenized (0.2 g/ml) in PBS (mmol/l: NaCl 136.9, Na_2HPO_4 8.1, KCl 2.7, KH_2PO_4 1.3, pH 7.5; SVA, Uppsala, Sweden) and incubated with [1,2(n)]-^{3}H-cortisone (33 nmol/l) (Amersham Pharmacia Biotech, Uppsala, Sweden) and NADPH (33 μmol/l) for 15 min (37°C; Tris 30 mmol/l, pH 7.2, EDTA 1 mmol/l). The reaction was stopped with perchloric acid and the samples were centrifuged at 1300 g for 3 min. ^{3}H-Cortisol and ^{3}H-cortisone in the supernatant were separated by HPLC (HP1100; Hewlett-Packard, Palo Alto, Calif., USA; 15 μl injection volume, 5 μm diameter C18 column; acetonitrile:water 28:72, 0.8 ml/min) [22]. Radioactivity was measured by adding scintillation cocktail (Ultima-Flo, Packard, Meriden, Conn., USA) to the effluent and passed through an on-line liquid scintillation spectrometer (Flow scintillation analyser, Packard).

Hepatic mRNA. Real-time PCR was used to quantify mRNA concentrations of 11β-HSD1, PEPCK and G6Pase (TaqMan, Applied Biosystems, Foster City, Calif., USA). Total RNA was prepared from frozen livers using RNAqueous (Ambion, Tex., USA) and treated with DNase (Ambion). The cDNA was prepared from all of the total RNA samples using the TaqMan reverse transcription reagent. The PCR was made with primer, probe, TaqMan Universal PCR Master Mix, and cDNA (each sample in triplicate). The ABI Prism 7700 Sequence Detection System (software version 1.6) was used for analysis. Results were normalized to endogenous control GAPDH mRNA concentrations (Applied Biosystems). GAPDH mRNA concentrations were compared with 18S rRNA and did not differ between groups.

In each case the amplicon includes a sequence corresponding to an exon border in the genome. The *11β-HSD1* probe and the forward primers for *PEPCK* and *G6Pase* span exon borders in the respective genes.

The following forward primers, reverse primers, and probes, respectively, were designed (melting temperature,°C; concentration, nmol/l):

11β-HSD1:

5′-agcagagcaatggcagcat (58, 300);
5′-gagcaatcataggctgggtca (59, 50);
5′FAM-cgtcatctcctccttggctgggaa-TAMRA (68, 100).

PEPCK:

5′-ggcggagcatatgctgatcc (61, 500);
5′-ccacaggcactagggaaggc (60, 50);
5′FAM-ccccgaaggcaagaagaaatacctggc-TAMRA (70, 200).

G6Pase:

5′-tcaacctcgtcttcaagtggatt (59, 300);
5′-gctgtagtagtcggtgtccagga (59, 300);
5′FAM-tgtttggacaacgcccgtattggtg-TAMRA (69, 100).

Blood chemistry. Glucose concentration was measured immediately upon sampling (Accutrend, Roche, Basle, Switzerland). The mice were not fasted during the experiment, since fasting induces hepatic PEPCK mRNA transcription [19].

Serum was prepared from blood obtained after cervical dislocation and left half an hour at 4°C before centrifugation for 10 to 15 min at 3000 g. The serum was stored at –70°C until analysis.

Serum insulin was analysed with rat insulin RIA (Linco, St. Louis, Mo., USA). Serum AST, ALT and ALP activity was measured with kinetic UV methods (Roche).

Serum and hepatic BVT.2733. Serum (10 µl) was diluted with 200 µl trifluoroacetic acid (1%) containing internal standard (carbamazepine; 0.05 µmol/l). Fifty microlitres of the diluted serum was injected on a Prospect on-line solid phase extraction system (Spark Holland, Emmen, The Netherlands) loaded with extraction cartridges (Waters Oasis, Milford, Mass., USA; 10*2 mm I.D.) coupled to liquid chromatography – tandem mass spectrometry detection (LC-MS-MS; Micromass Quattro II, Manchester, UK).

Liver samples were homogenized in PBS (0.2 g/ml). Proteins were precipitated from the homogenate (0.1 ml) by acetonitrile (0.4 ml) with internal standard (carbamazepine; 0.5 µmol/l). After 30 min at room temperature the samples were centrifuged for 5 min (18200 g), the supernatant (50 µl) was diluted with 150 µl trifluroacetic acid (1%), and 50 µl of the dilute was injected on the solid phase extraction system coupled to LC-MS-MS as described for the serum samples.

The instrument was set up with an auto-injector (Gilson ASPEC XL, Middleton, Wiss., USA) and LC-pumps (Shimadzu 10AD, Kyoto, Japan). The analytes were separated on an HPLC-column (Zorbax SB-18, 2.1*50 mm, 5 µm; Agilent Technologies, Wilmington, Del., USA). The mobile phase consisted of (i) 2% acetonitrile and 98% formic acid (8 mmol/l), and (ii) 95% acetonitrile and 5% formic acid (133 mmol/l). The flow rate was 0.3 ml/min with a mobile phase gradient from 10 to 100% of (B) over a period of 3 min. Detection was achieved by electrospray in positive ion mode and multiple reaction monitoring for the transition in mass per charge 429 to 140 for BVT.2733 and mass per charge 237 to 194 for carbamazepine. BVT.2733 (serum: 0.05–50 µmol/l; liver: 1–100 µmol/l) standards were made in mouse serum or in pooled mouse liver homogenate (vehicle).

Food intake and body weight. Animals and fodder were weighed for 3 days before and every morning after pump implantation.

Compound. 3-chloro-2-methyl-N-{4-[2-(4-methyl-1-piperazinyl)-2-oxoethyl]-1,3-thiazol-2-yl} benzenesulfonamide (BVT.2733) (Biovitrum, Stockholm, Sweden) was dissolved in aqueous (2-β-hydroxypropyl)-cyclodextrin (12%; Fluka, Steinheim, Germany).

Statistics. The Mann-Whitney non-parametric test (SPSS 10.1, Chicago, IL., USA) was used for statistical evaluation of the results. Results are expressed as means ± SEM.

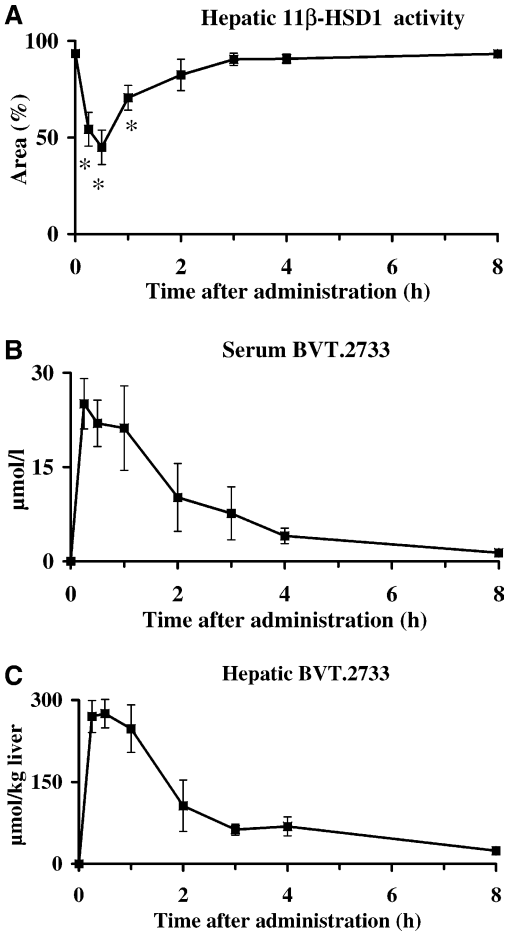

Fig. 1A–C. Effect of a single BVT.2733 (100 mg·kg^{-1}; p.o.; n=4) administration in KKA^{y} mice. **A** Hepatic 11β-HSD1 activity (^{3}H-cortisol (^{3}H-cortisone+^{3}H-cortisol)$^{-1}$) (Area, %). **B** Serum BVT.2733 concentrations (µmol/l). **C** Hepatic BVT.2733 levels (µmol/kg liver). Statistical difference (p) from control is: *p<0.05

Results

Acute administration. Administration of a single bolus dose of BVT.2733 (100 mg·kg^{-1}; p.o.) resulted in an inhibition of hepatic 11β-HSD1 activity (Fig. 1A). The maximal inhibition of hepatic 11β-HSD1 activity occurred after approximately half an hour and correlated with the maximum concentration of BVT.2733 both in liver and blood (Fig. 1B, C).

7-Day administration. BVT.2733 was administered as a continuous subcutaneous infusion by osmotic minipumps (167 mg·kg^{-1}·day^{-1}). After 7 days of treatment, serum BVT.2733 concentration was 7.4±2.6 µmol/l (n=22) and liver concentrations were 24.1± 6.2 µmol/kg (n=10). At that time, the hepatic PEPCK mRNA concentration was decreased to 75±17% (n=10; p<0.05) and G6Pase mRNA concentration to 55±51% (n=10; p=0.089) of the concentrations in vehicle treated animals (Fig. 2). However, hepatic 11β-HSD1 mRNA concentration was not altered

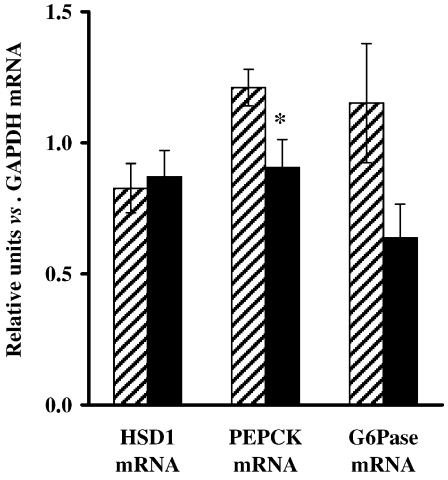

Fig. 2 Effect of 7-day BVT.2733 (167 mg·kg^{-1}·day^{-1}; s.c. osmotic pump; n=10) administration in KKA^y mice on hepatic mRNA concentrations of 11β-HSD1, PEPCK and G6Pase. Results are given as relative units vs GAPDH mRNA. Statistical difference (p) from vehicle is: *p<0.05. (*hatched bars*) Vehicle and (*solild bars*) BVT.2733

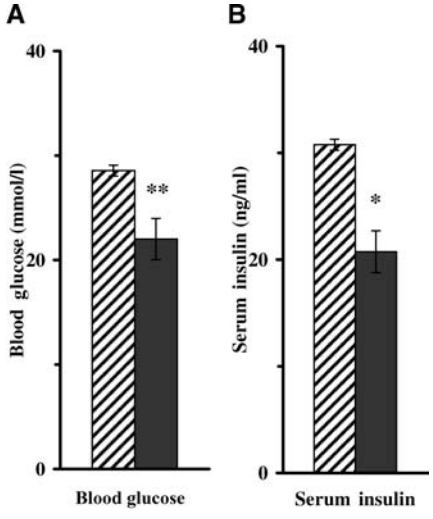

Fig. 3A, B. Effect of 7-day BVT.2733 (167 mg·kg^{-1}·day^{-1}; s.c. osmotic pump) administration in KKA^y mice. **A** Blood glucose concentrations (BVT.2733: n=17, vehicle: n=15). **B** Serum insulin concentrations (BVT.2733: n=19, vehicle: n=20). Statistical difference (p) from vehicle is: *p<0.05, **p<0.01. (*hatched bars*) Vehicle and (*solid bars*) BVT.2733

(Fig. 2). Treatment with BVT.2733 lowered blood glucose and insulin concentrations. The blood glucose concentrations were 77±12% (n=17; p<0.01) of control and serum insulin concentrations were 67±26% (n=20; p<0.05) of control (Fig. 3A, B). Daily food intake, body weight, and serum AST, ALT and ALP were all unaffected by the BVT.2733 treatment.

Discussion

We describe that treatment with a selective inhibitor of 11β-HSD1, BVT.2733, results in lowering of blood glucose and serum insulin concentrations in a hyperglycaemic and hyperinsulinaemic mouse. BVT.2733 was administered with osmotic mini-pumps to obtain steady-state compound blood concentrations and enzyme inhibition. A pilot study was carried out to show that the 11β-HSD1 enzyme was inhibited in vivo. The results show that the inhibition closely reflects the serum and liver BVT.2733 concentrations. Thus, dilution of the tissue homogenate in the enzyme activity assay resulted in underestimation of the reversible inhibition in vivo.

It has been shown that *11β-HSD1* knock-out mice fed a high-fat diet have lower blood glucose concentrations than wild-type control mice [19, 21]. This difference however, was observed after the animals had been on the diet for 8 weeks. In our study, pharmacological inhibition of 11β-HSD1 in KKA^y mice lowered blood concentrations levels significantly (p<0.01) already within 7 days and was not paralleled by changes in daily food intake or body weight. Thus, both the previous gene disruption and the present BVT.2733 inhibition of 11β-HSD1 resulted in reduced blood glucose concentrations.

Administration of BVT.2733 reduced hepatic concentrations of mRNA encoding PEPCK and G6Pase – the rate-limiting enzymes for gluconeogenesis and the former only regulated on the mRNA level [6]. This is consistent with observations in *11β-HSD1* knock-out mice and supports the mechanistic rationale that glucocorticoids are permissive for excessive hepatic glucose production. In contrast to pharmacological inhibition, reductions of hepatic mRNA concentrations of PEPCK and G6Pase in *11β-HSD1* knock-out mice were observed only after fasting [19, 21]. Thus, it is likely, that our results underestimate the reduction in hepatic mRNA concentrations of PEPCK and G6Pase, and that pharmacological 11β-HSD1 inhibition possibly results in even larger reductions after a fasting period. Importantly, BVT.2733 administration for 7 days did not alter hepatic 11β-HSD1 mRNA concentration. The absence of any compensatory transcription of hepatic 11β-HSD1 mRNA after selective pharmacological inhibition of the enzyme is in agreement with a previous report on the use of the non-selective inhibitor glycyrrhizic acid in rats [23]. Thus, inhibition of 11β-HSD1 offers an effective pharmacological intervention that is likely to yield sustained reduction of glucocorticoid-inducible hepatic gluconeogenic enzymes.

The non-selective 11β-HSD1 inhibitor carbenoxolone has been used in a clinical study [18]. Then, glucose infusion rate was higher than control in a euglycemic hyperinsulinaemic clamp. Improved hepatic insulin sensitivity and decreased glucose production were also shown. However, the dose of the non-selective inhibitor (carbenoxolone) was limited by effects on the type 2 isoform of 11β-HSD in the kidney, inhibition of which yields hypertension [18].

BVT.2733 is a new, selective inhibitor for the 11β-HSD1 enzyme (with a K_i of 1 μmol/l) over the type 2 isoenzyme (no inhibition at 200 μmol/l). The affinity of BVT.2733 for other enzymes and receptors has not been investigated. However, six structurally related compounds have been subjected to a selectivity panel and were found to be devoid of any significant affinity ($p>0.05$ at 1 μmol/l) to a broad panel of enzymes and receptors, including the estrogen, glucocorticoid, progesterone, and androgen receptors (Cerep, Celle l'Evescault, France). Thus, BVT.2733 is from a class of selective 11β-HSD1 inhibitors. In support of the selectivity of BVT.2733, the results show that BVT.2733 did not alter common liver function marker enzymes such as AST, ALT, or ALP, indicating that the effects on hepatic glucose production can be ascribed to 11β-HSD1 inhibition.

In conclusion, administration of a selective 11β-HSD1 inhibitor lowered hepatic PEPCK and G6Pase mRNA, blood glucose and serum insulin concentrations in hyperglycaemic KKA^y mice. These results suggest that a selective inhibitor of human 11β-hydroxysteroid dehydrogenase type 1 can become a new approach for lowering blood glucose concentrations in Type II diabetes [24].

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