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RESEARCH PAPER

Emodin, a natural product, selectively inhibits 11β-hydroxysteroid dehydrogenase type 1 and ameliorates metabolic disorder in diet-induced obese mice

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emodin; 11β-hydroxysteroid dehydrogenase type 1; diet-induced obese mice; insulin resistance; metabolic syndrome; type 2 diabetes

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BACKGROUND AND PURPOSE
11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an attractive therapeutic target of type 2 diabetes and metabolic syndrome. Emodin, a natural product and active ingredient of various Chinese herbs, has been demonstrated to possess multiple biological activities. Here, we investigated the effects of emodin on 11β-HSD1 and its ability to ameliorate metabolic disorders in diet-induced obese (DIO) mice.

EXPERIMENTAL APPROACH
Scintillation proximity assay was performed to evaluate inhibition of emodin against recombinant human and mouse 11β-HSDs. The ability of emodin to inhibit prednisone- or dexamethasone-induced insulin resistance was investigated in C57BL/6J mice and its effect on metabolic abnormalities was observed in DIO mice.

KEY RESULTS
Emodin is a potent and selective 11β-HSD1 inhibitor with the IC_{50} of 186 and 86 nM for human and mouse 11β-HSD1, respectively. Single oral administration of emodin inhibited 11β-HSD1 activity of liver and fat significantly in mice. Emodin reversed prednisone-induced insulin resistance in mice, whereas it did not affect dexamethasone-induced insulin resistance, which confirmed its inhibitory effect on 11β-HSD1 *in vivo*. In DIO mice, oral administration of emodin improved insulin sensitivity and lipid metabolism, and lowered blood glucose and hepatic PEPCK, and glucose-6-phosphatase mRNA.

CONCLUSIONS AND IMPLICATIONS
This study demonstrated a new role for emodin as a potent and selective inhibitor of 11β-HSD1 and its beneficial effects on metabolic disorders in DIO mice. This highlights the potential value of analogues of emodin as a new class of compounds for the treatment of metabolic syndrome or type 2 diabetes.

Abbreviations
11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; DIO, diet-induced obese; G6Pase, glucose-6-phosphatase; GC, glucocorticoid; GR, glucocorticoid receptor; NEFA, non-esterified free fatty acid; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; SPA, scintillation proximity assay

Introduction

Insulin resistance plays a key role in the pathogenesis of type 2 diabetes, and is often associated with a group of metabolic disorders, such as hyperglycaemia, hypertension, dyslipidaemia, obesity and atherosclerotic vascular disease (DeFronzo and Ferrannnini, 1991). Glucocorticoids antagonize the action of insulin and induce insulin resistance when in excess (Livingstone *et al.*, 2000; Masuzaki *et al.*, 2001; Beauregard *et al.*, 2002). The high circulating glucocorticoid level in Cushing's syndrome leads to visceral obesity and several clinical features associated with insulin resistance (Beauregard *et al.*, 2002). Pharmacological blockade of glucocorticoid action by glucocorticoid receptor (GR) antagonist can ameliorate diabetes and insulin resistance, which highlights the importance of glucocorticoids in the development of type 2 diabetes and metabolic syndrome (Wang *et al.*, 2006).

The action of glucocorticoids on target tissue is not only dependent on the circulating levels, but is regulated in a tissue-specific manner by the enzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2 (Draper and Stewart, 2005). 11β-HSD1 is a low-affinity, NADP(H)-dependent dehydrogenase/oxoreductase that functions predominantly as an oxoreductase in intact cells, organs and *in vivo*. It is highly expressed in liver, gonad, adipose tissue and brain, and amplifies local glucocorticiod action by converting cortisone into cortisol in humans, and 11-dehydrocorticosterone into corticosterone in rodents (Seckl and Walker, 2001). 11β-HSD2 is predominantly expressed in aldosterone target cells such as kidney and colon, and catalyses the opposite reaction, thereby preventing excessive activation of the mineralocorticoid receptor and sequelae including sodium retention, hypokalaemia and hypertension.

Accumulating evidence suggests that 11β-HSD1 plays an important role in the development of obesity, insulin resistance and type 2 diabetes. 11β-HSD1 mRNA expression or activity is specifically increased in liver or fat of Zucker rat, ob/ob mice and db/db mice, and may contribute to the phenotype of type 2 diabetes (Masuzaki *et al.*, 2001). Mice with transgenic overexpression of 11β-HSD1 selectively in adipose tissue had increased intra-adipose glucocorticoid concentrations with no change in plasma levels, and exhibited visceral obesity, insulin resistance, hyperglycaemia and hyperlipidaemia (Masuzaki *et al.*, 2001). In contrast, 11β-HSD1-deficient mice exhibited enhanced glucose tolerance, attenuated gluconeogenic responses, increased insulin sensitivity, improved lipid and lipoprotein profile and resistance to hyperglycaemia and weight gain induced by a high-fat diet (Kotelevtsev *et al.*, 1997; Morton *et al.*, 2001). In humans, increased 11β-HSD1 activity or mRNA has been observed in subcutaneous fat, which was positively correlated with obesity, insulin resistance and other features of metabolic disorders (Sandeep *et al.*, 2005). The non-selective 11β-HSD1 inhibitor carbenoxolone increases hepatic insulin sensitivity in healthy men and non-obese type 2 diabetic patients, as measured by an increase in glucose infusion rate during euglycaemic hyperinsulinaemic clamp (Andrews *et al.*, 2003). Carbenoxolone and several selective inhibitors of 11β-HSD1 have also been shown to ameliorate hyperglycaemia, and improve glucose tolerance and insulin sensitivity in rodent models of diabetes or metabolic syndrome (Alberts *et al.*, 2003; Hermanowski-Vosatka *et al.*, 2005). Therefore, all these findings suggest that the pharmacological inhibition of 11β-HSD1 could provide a very attractive therapy of type 2 diabetes or metabolic diseases.

Due to the unacceptable side effects caused by the inhibition of 11β-HSD2, including sodium retention and hypertension, the ideal inhibitors must be selective for 11β-HSD1 rather than 11β-HSD2. Several natural compounds and their derivatives including glycyrrhetinic acid and carbenoxolone have been investigated as 11β-HSD1 inhibitors, but in general they are non-selective. To discover new selective 11β-HSD1 inhibitors from natural products, we performed a screening of our phytocompound collection based on a scintillation proximity assay (SPA) against recombinant human and mouse 11β-HSDs. A series anthraquinone compounds had been shown to inhibit 11β-HSD1 of both humans and mice. Among those compounds, emodin (1,3,8-trihydroxy-6-methylanthraquinone), an anthraquinone derivative isolated mainly from the root and rhizome of *Rheum palmatum*, has been identified as the most potent selective 11β-HSD1 inhibitor. Therefore, in the present study, emodin was firstly identified as a selective 11β-HSD1 inhibitor based on both its *in vitro* and *in vivo* activities, and, secondly, its effect on metabolic abnormalities was investigated in diet-induced obese (DIO) mice with insulin resistance and dyslipidaemia.

Methods

Animals

C57BL/6J male mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China) and maintained on a 7:00 h–19:00 h light schedule with an *ad libitum* diet of standard lab chow, unless otherwise specified. For DIO mice study, the

C57BL/6J male mice were fed with a high-fat diet (60 kcal% fat, 20 kcal% protein and 20 kcal% carbohydrate, Cat. D12492i, Research Diet, New Brunswick, NJ, USA). Animal experiments were approved by the Animal Care and Use Committee, Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Construction of stably transfected cells

The full-length cDNAs of human or murine 11β-HSD1 and 11β-HSD2 were isolated from the cDNA libraries provided by NIH Mammalian Gene Collection and cloned into pcDNA3 expression vector by PCR. HEK-293 cells were transfected with each cDNA expression construct via lipofactamine technology. Transfected cells were selected by cultivation in the presence of 700 $\mu g \cdot mL^{-1}$ of G418. Non-resistant cells were removed by replacing the cell culture medium every other day for 12–14 days. The single surviving colony was picked up and expanded. The protein expression of human or mouse 11β-HSD1 and 11β-HSD2 was confirmed, respectively, by Western blot. The enzymes of 11β-HSDs were purified, respectively, according to the method previously described (Mundt *et al.*, 2005).

Measurement of 11β-HSD1 and -HSD2 activity in vitro

The SPA was used to screen for inhibitors of 11β-HSDs (Mundt *et al.*, 2005), with the microsome fractions prepared from the HEK-293 cells stably transfected with either human or mouse 11β-HSD1 or 11β-HSD2 as the enzyme source. Briefly, different concentrations of compound were added to 96-well microtitre plates, followed by the addition of 80 µL of 50 mM HEPES buffer, pH 7.4 containing 25 nM [1,2-(n)^{3}H]-cortisone and 1.25 mM NADPH (for 11β-HSD1 assay) or 12.5 nM [1,2,6,7-(n)^{3}H]-cortisol and 0.625 mM NAD^+(for 11β-HSD2 assay). Reactions were initiated by the addition of 11β-HSD1 or 11β-HSD2, enzyme preparation as microsome fractions from HEK293 cells in a final concentration of 80 $\mu g \cdot mL^{-1}$ for 11β-HSD1, and 160 $\mu g \cdot mL^{-1}$ for 11β-HSD2, respectively. After a 60 min incubation at 37°C, the reaction was stopped by the addition of 35 µL of 10 $mg \cdot mL^{-1}$ protein A-coated yttrium silicate beads suspended in SuperBlock Blocking Buffer with 3 $\mu g \cdot mL^{-1}$ of murine monoclonal cortisol antibody and 314 µM glycyrrhetinic acid. The plates were incubated under plastic film on an orbital shaker for 120 min at room temperature before counting. The amount of [^{3}H]-cortisol generated in 11β-HSD1 enzyme reaction or remaining from the 11β-HSD2 enzyme reaction was captured by the beads and determined in a microplate liquid scintillation counter. The % inhibition was calculated relative to a non-inhibited control. Data were obtained from at least three independent experiments. IC_{50} values were calculated from concentration–response curves by a non-linear regression analysis using Prism Version 4.

Molecular modelling

The program DOCK4.0 (Kuntz, 1992; Ewing and Kuntz, 1997) was employed for the docking study. The starting structure was PDB entry 2IRW (Patel *et al.*, 2007), and residues around the ligand in this structure at a radius of 5 Å were isolated for constructing the grids of docking. During the docking calculations, Kollman-all-atom charges (Weiner *et al.*, 1986) were assigned to the protein, and Gasterger–Hückel charges (Gasteiger and Marsili, 1980) were assigned to the small molecules. Conformational flexibility of the small molecules was implemented in the docking search. The ligand–receptor binding energy was approximately set to be the sum of the van der Waals and electrostatic interaction energies. After an initial evaluation of the orientation and scoring, a grid-based minimization was carried out for the ligand to locate the nearest local energy minimum within the receptor binding site. Position and conformation of each docked molecule were optimized by using the single anchor search and torsion minimization method.

Acute administration in normal mice

To evaluate the activity of acute administration of emodin, C57 BL/6J mice deprived of food overnight were administered emodin (100 or 200 $mg \cdot kg^{-1}$) or vehicle (0.5% carboxymethylcellulose; CMC) p.o. Two hours later, animals were killed by cervical dislocation, and the liver and mesenteric fat were isolated immediately, washed in ice-cold PBS, frozen in liquid nitrogen and stored at –80°C. The liver and mesenteric fat were homogenized (0.1 $g \cdot mL^{-1}$) in cold homogenization buffer (20 mM Na_2HPO_4, 5% glycerol, 1 mM EDTA, pH 7.0), and 10 µg of liver homogenates or 30 µg mesenteric fat homogenates was used to analyse the 11β-HSD1 activity by SPA, as previously described.

Effect of emodin on prednisone- or dexamethasone-induced insulin-resistant mice

Male C57BL/6J mice (8 weeks old) were randomly assigned to six groups based on body weight. The experimental groups and respective treatment were as follows: (i) control – 0.5% CMC; (ii) prednisone acetate – 100 $mg \cdot kg^{-1}$; (iii) prednisone acetate (100 $mg \cdot kg^{-1}$) plus emodin (100 $mg \cdot kg^{-1}$); (iv) prednisone acetate (100 $mg \cdot kg^{-1}$) plus emodin (200 $mg \cdot kg^{-1}$); (v) dexamethasone (4 $mg \cdot kg^{-1}$); and (vi) dexamethasone (4 $mg \cdot kg^{-1}$) plus emodin

(200 mg·kg^{-1}). Prednisone or dexamethasone was administered by oral gavage twice daily to induce a state of glucocorticoid excess and insulin resistance in mice. Emodin was administered orally twice daily 1 day before, and then at the same time as prednisone or dexamethasone. After 14 days of treatment, insulin tolerance was determined in mice deprived of food overnight (0.5 U·kg^{-1} insulin administered by an i.p. injection) to investigate the effect of emodin on prednisone- or dexamethasone-induced insulin resistance.

Effect of emodin in DIO mice

C57BL/6J male mice (3–4 weeks) were fed a formulated research diet containing 60% of the calories from fat for 12 weeks before, and throughout the duration of the experiment. DIO mice were assigned to three groups and subjected to gavage treatment twice per day with vehicle (0.5% CMC), emodin 50 or 100 mg·kg^{-1}, respectively, for 35 days. Fasting blood glucose values and initial body weights were comparable between groups. The blood glucose levels were measured via blood drops obtained by clipping the tail of the mice using a ONE TOUCH BASIC plus Glucose Monitor (Lifescan, Milpitas, CA, USA), unless otherwise specified. The food intake and body weight of the animals were recorded every 3 days. Glucose tolerance test was determined in mice deprived of food for 5 h (2 g·kg^{-1} glucose administered by gavage) at day 24 of the treatment. The blood samples were collected via the retro-orbital sinus, and the serum glucose and insulin concentrations were measured with an enzymatic colorimetric method and insulin ELISA kit, respectively. An insulin tolerance test was performed in the 5 h-fasted mice (0.5 U·kg^{-1} insulin administered by an i.p. injection) at day 28 of the treatment. On the last day of treatment, 5 h-fasted mice were anaesthetized with an i.p. injection of sodium pentobarbital (40 mg·kg^{-1}). Serum was collected for determination of insulin, triacylglycerol, cholesterols and non-esterified free fatty acid (NEFA) concentration. The liver and different fat pads including epididymal fat, mesenteric fat, perirenal fat and subcutaneous fat were dissected, weighed, immediately frozen in liquid nitrogen and stored at –80°C.

Quantification of mRNA

Real-time PCR was used to quantify mRNA levels of 11β-HSD1 in liver and fat tissues. PEPCK and G6Pase mRNA levels in liver were also determined. Total RNA was extracted from frozen livers and fat tissues using Trizol reagent. The mRNA was quantified using a BIO-RAD (Bio-rad Laboratories Inc., Hercules, CA, USA) SYBR Green Supermix and an MJ research DNA engine Opticon2. Primers were as follows: for 11β-HSD1, 11β-HSD1F (GGAGGAGAT GACGGCAAT), and 11β-HSD1R (CC TTGAACTCG GAGCAGC); for PEPCK, PEPCKF (TGATGACTGTC TTGCTTTCG) and PEPCKR (GCATA ACGGTCTGG ACTTCT); for G6Pase, G6PaseF (CTCGCTATCTCCA AGTGAAT) and G6PaseR (TGCTGTAGTAGTCGGT GTCC); for β-actin, β-actinF (TGCTGTCCCTGTAT GCCTCTG) and β-actinR (TTGATGTCACGCACGAT TTCC). The Opticon Monitor (software version 1.11) was used for analysis. Results were normalized to endogenous control β-actin mRNA expressions.

Data analysis

The data are presented as mean with the SEM as indicated. All data were normally distributed. To determine treatment effects, statistical analysis was performed by use of a two-tailed unpaired *t*-test. *P* values of less than 0.05 were considered statistically significant.

Materials

Emodin and other compounds were purchased from Nanjing Zelang Medical Technology Co. Ltd. (Qixia District, Nanjing, Jiang Su, China). The pcDNA expression vector and Trizol Reagent (Cat. no. 15596-018) were purchased from Invitrogen (Carlsbad, CA, USA). [1,2-(n)^{3}H]- cortisone (Cat. TRK1075) was from Amersham (Buckinghamshire, UK). [1,2,6,7-(n)^{3}H]- cortisol (Cat. NET396) was from PerkinElmer (Boston, MA, USA). SPA beads (Cat. RPN143) were from GE (Piscataway, NJ, USA). SuperBlock Blocking Buffer (Cat. 37515) was from Pierce (Rockford, IL, USA). The murine monoclonal cortisol antibody (Cat. P01-92-94M-P) was from East Coast Biologics (North Berwick, ME, USA). Glycyrrhetinic acid was from Sigma (St. Louis, MO, USA). The M-MLV reverse transcriptional enzyme (Cat. M170A) was from Promega (Madison, WI, USA). All the primers were synthesized by Sangon Corporation (Song Jiang, Shanghai, China). SYBR Green Supermix (cat.170-8880) was from Bio-Rad. The high-fat forage (Cat. D12492i) was from Research Diet (Research Diets Inc., New Brunswick, NJ, USA). Blood glucose values were measured using a One-Touch Basic Glucose Monitor (Lifescan, Milpitas, CA, USA). Serum insulin was analysed with a mice insulin ELISA kit (cat. 90080, Crystal Chem, Downers Grove, IL, USA). Serum NEFA was determined with an enzymatic colorimetirc method using oleic acid as a standard (NEFA C, cat. 994-75409, Wako Chemicals, Neuss, Germany). Serum triacylglycerols and cholesterols were analysed with an enzymatic colorimetric method (Hitachi 7060 Biochemical Autoanalyser, Chlyoda-Ku, Tokyo, Japan).

Table 1

In vitro inhibitory effects of anthraquinone compounds on 11β-HSD1 and 2

Compound	Mouse 11β-HSD1	Human 11β-HSD1	Mouse 11β-HSD2	Human 11β-HSD2
Emodin	86	186	>1 mM	>1 mM
Aloe-emodin	98	879	>1 mM	ND
Rhein	2840	>10 000	>1 mM	ND
Rheochrysidin	81	542	>1 mM	ND
3-Methylchrysazin	400	3540	>1 mM	ND

The values shown are IC_{50} (nmol·L^{-1}). ND, not determined.

Results

Emodin selectively inhibited 11β-HSD1 activity in vitro

The potency and selectivity of a series anthraquinone compounds on the inhibition of mouse or human 11β-HSD1 or 2 were determined by SPA. IC_{50} values are presented in Table 1. Emodin, aloe-emodin and rheochrysidin showed a strong inhibitory effect on recombinant mouse 11β-HSD1 with IC_{50} of 86, 98 and 81 nM, respectively. Emodin also inhibited human 11β-HSD1 with IC_{50} of 186 nM, whereas aloe-emodin and rheochrysidin were less potent with the IC_{50} of 879 and 542 nM, respectively. The other two anthraquinone compounds, rhein and 3-methylchrysazin, exhibited much weaker inhibitory effects on both mouse and human 11β-HSD1. All of the five anthraquinone compounds showed good selectivity for mouse 11β-HSD2 with an IC_{50} > 1 mM, and emodin did not have a significant inhibitory effect on human 11β-HSD2. Therefore, a series anthraquinone compounds were identified as selective 11β-HSD1 inhibitors, emodin being the most potent.

Molecular modelling of emodin and 11β-HSD1

To explain the interaction mode of emodin to human 11β-HSD1, molecular docking simulation was performed employing the program DOCK4.0 based on the X-ray crystal structure of the 11β-HSD1 complex (PDB entry 2IRW). This complex structure is composed of human 11β-HSD1, a synthetic inhibitor with high activity, and a co-substrate nicotinamide adenine dinucleotide phosphate (NADP). The emodin was docked into the binding site flexibly; meanwhile, the structure of 11β-HSD1 and NADP was fixed. The conformation with the lowest interaction energy was taken out for further analysis.

In the initial crystal structure, hydrogen bonds provide strong interactions between the ligand and the protein, as well as its co-substrate NADP. The carbonyl group of the ligand forms two hydrogen bonds with Tyr183 and Ser170. Interestingly, the docking results showed that emodin also formed strong hydrogen bonds with the receptor, as shown in Figure 1. The hydroxyl on C4 formed hydrogen bonds with Ser170, and the carbonyl group on C8 formed two hydrogen bonds with Ser170 and Tyr183 (Figure 1B). However, emodin did not form a hydrogen bond with NADP as did the ligand in the crystal structure. Instead, emodin formed hydrophobic contacts with the NADP (Figure 1B). Furthermore, residues Leu126, Val227 and Tyr177 were involved in the hydrophobic contacts with emodin (Figure 1B).

Emodin inhibited 11β-HSD1 activity in vivo

The *in vivo* efficacy of emodin at inhibiting 11β-HSD1 activity was evaluated in C57BL/6J mice. Two hours after p.o. administration of 100 or 200 mg·kg^{-1} emodin, the mice were killed, and the liver and mesenteric fat were removed and assayed for 11β-HSD1 activity. As shown in Figure 2, oral administration of 100 or 200 mg·kg^{-1} of emodin significantly inhibited liver 11β-HSD1 enzymatic activity by 17.6 and 31.3% (Figure 2A) and mesenteric fat 11β-HSD1 enzymatic activity by 21.5 and 46.7% (Figure 2B), respectively. The results demonstrate that emodin inhibits 11β-HSD1 activity *in vivo*.

Emodin antagonized insulin resistance induced by glucocorticoids

It is well documented that prolonged exposure to elevated glucocorticoid levels produces insulin resistance, a hallmark of diabetes mellitus. Dexamethasone is a synthetic active glucocorticoid, which has a strong affinity for the GR, whereas prednisone is a synthetic cortisone analogue, which has little affin-

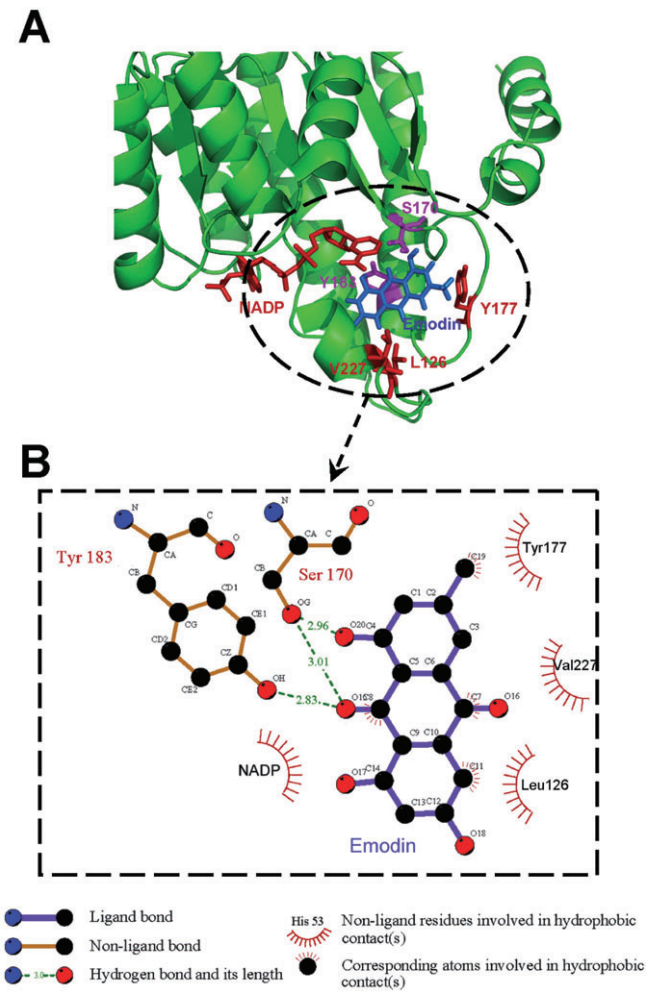

Figure 1

Molecular modelling of emodin and 11β-HSD1. Molecular docking simulation was performed employing the program DOCK4.0, based on the X-ray crystal structure of 11β-HSD1 complex (PDB entry 2IRW). The complex structure (A), hydrophobic contacts among 11β-HSD1, emodin and NADP (B).

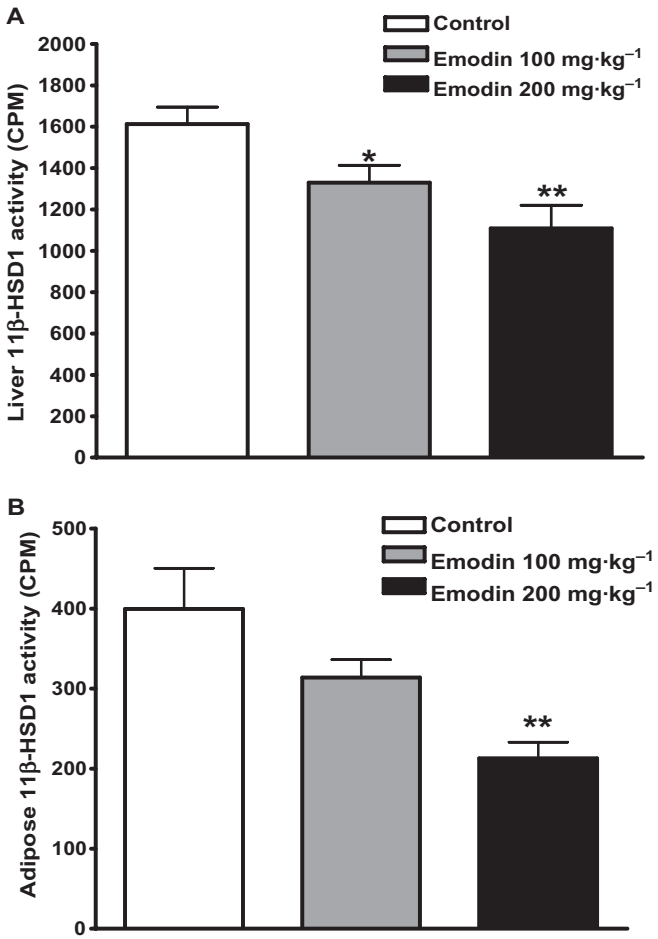

Figure 2

Oral administration of emodin inhibited hepatic 11β-HSD1 activity in C57BL/6J mice. Emodin was orally administered to male C57BL/6J mice, and analysis of 11β-HSD1 activity in 10 μg liver homogenates (Figure 2A) and 30 μg mesenteric fat homogenates (Figure 2B) was conducted with SPA at 2 h post-dose. Data are expressed as mean ± SEM for $n = 10$ mice. *$P < 0.05$; **$P < 0.01$ versus control mice.

ity for the GR. However, prednisone can be catalysed by the liver 11β-HSD1 to convert it into its active metabolite, prednisolone, which has relatively high glucocorticoid activity. The insulin tolerance test showed that treatment of C57BL/6J mice with dexamethasone or prednisone for 14 days reduced the glucose-lowering effect in response to the insulin challenge, indicating the presence of insulin resistant (Figure 3). When concurrently treated with 100 or 200 mg·kg^{-1} emodin, the glucose-lowering effects after insulin injection were increased in prednisone-treated mice, which suggests improved insulin sensitivity. In contrast, the insulin resistance induced by dexamethasone was not improved by the concurrent treatment with 200 mg·kg^{-1} emodin (Figure 3). These results indicate that emodin can reverse prednisone-, but not dexamethasone-induced insulin resistance in mice, which confirms its inhibitory effect on 11β-HSD1 *in vivo*.

Emodin improved metabolic abnormalities of DIO mice

C57BL/6J mice fed a high-fat diet developed moderate obesity, mild hyperglycaemia, dyslipidaemia and insulin resistance. Emodin (100 mg·kg^{-1}) administered by oral gavage b.i.d. for 7 days reduced fasting glucose concentrations to 77.2% of the vehicle control mice, and these remained significantly lower ($P < 0.01$) throughout the treatment period (Figure 4A). After 24 days of treatment with emodin, the DIO mice exhibited a significant reduction in blood glucose levels at all time-points following oral glucose challenge (Figure 4B) ($P < 0.05$ to $P < 0.01$). This was accompanied by a reduction in

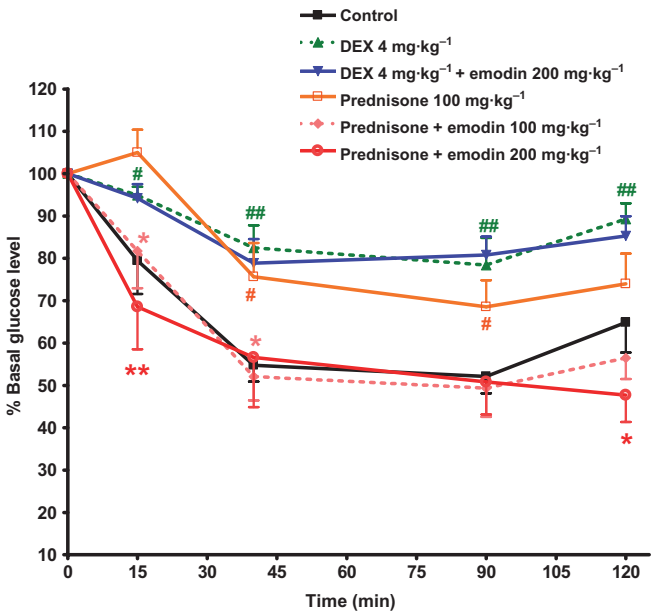

Figure 3

Emodin antagonized insulin resistance induced by glucocorticoids. Mice were treated as described in Methods. The insulin tolerance test was conducted in mice deprived of food overnight (0.5 U·kg^{-1} insulin administered by an i.p. injection) at day 14 of treatment. Values are expressed as mean ± SEM for n = 8 mice. #P < 0.05; ##P < 0.01 versus control mice. *P < 0.05; **P < 0.01 versus prednisone-treated mice.

serum insulin concentrations at 15, 30 and 60 min after glucose loading in the 100 mg·kg^{-1} emodin-treated mice (Figure 4C). Treatment with emodin for 28 days also evoked a significantly greater reduction in blood glucose values 40 and 90 min after insulin injection (P < 0.05 to P < 0.01), indicating an improved insulin tolerance in emodin-treated DIO mice (Figure 4D). Moreover, the serum insulin level was also significantly reduced, to 66.2% of control mice, after 35 days of treatment with 100 mg·kg^{-1} emodin (P < 0.05, Figure 4E).

Emodin also improved the lipid profiles in DIO mice. After 35 days of treatment with 100 mg·kg^{-1} emodin, the serum triglyceride and total cholesterol levels were significantly reduced by 19.3 and 12.5% (P < 0.05), respectively, compared with vehicle control mice (Figure 5A,B). Emodin (100 mg·kg^{-1}) also caused a 22.7% reduction of NEFA level, although this did not reach statistical significance (P = 0.077) (Figure 5C).

Chronic treatment with emodin lowered body weight and appetite in DIO mice. DIO mice treated with 100 mg·kg^{-1} emodin showed a steady decline in body weight that was significantly different from vehicle-treated animals from day 18 of the treatment; their body weights were reduced by 13.9% (P < 0.01) at the end of treatment (Figure 6A). Emodin also affected the animals' feeding behaviour, resulting in a 17% reduction in food intake compared with the vehicle-treated animals (P < 0.05; Figure 6B). Furthermore, it caused a preferential reduction in mesenteric fat pad and perirenal fat pad weights by 29 and 47%, respectively. The subcutaneous fat weight in emodin-treated DIO mice was reduced compared with vehicle-treated control mice (Figure 6C), but it essentially had no effect on epididymal fat weight (data not shown).

Emodin suppressed 11β-HSD1 activity and reduced the mRNA levels of gluconeogenic genes in DIO mice

The enzymatic activity of 11β-HSD1 in liver and adipose tissues was measured 35 days after the treatment of DIO mice with 100 mg·kg^{-1} emodin. A significant decrease in 11β-HSD1 activity was observed in both the liver and mesenteric adipose tissues of emodin-treated DIO mice (P < 0.01). The 11β-HSD1 activity in liver and mesenteric adipose tissues was decreased by 53.5 and 41.2% (Figure 7A), respectively, whereas no significant change in 11β-HSD1 mRNA expression was observed (Figure 7B). Treatment of DIO mice with 100 mg·kg^{-1} emodin for 35 days significantly reduced hepatic PEPCK and G6Pase mRNA to levels 25.4 and 36.5% less than that of vehicle control mice (P < 0.05; Figure 7C).

Discussion

Emodin, a natural product and active ingredient of various Chinese herbs, has been demonstrated to possess multiple biological activities, including antitumour, antibacterial (Wang and Chung, 1997), anti-inflammatory (Chang *et al.*, 1996) and immunosuppressive effects(Huang *et al.*, 1992). Recent studies have shown that emodin could be a potential drug for the therapy of several proliferative diseases, such as liver cirrhosis (Woo *et al.*, 2002), diabetic nephropathy (Wang *et al.*, 2006), atherosclerosis (Heo *et al.*, 2008) and tumours (Huang *et al.*, 2007). Although a hypoglycaemic and hypolipidaemic effect of emodin had been reported in STZ-induced dyslipidaemic–diabetic rats (Zhao *et al.*, 2008), the effects of emodin on metabolic abnormalities, especially insulin resistance and the molecular mechanisms involved, have not been thoroughly studied. Our study shows for the first time that emodin is a potent selective 11β-HSD1 inhibitor and can ameliorate metabolic disorders in DIO mice.

11β-HSD1 is highly expressed in liver and adipose tissue, where it plays key role in the regulation of the local generation of active glucocorticoids

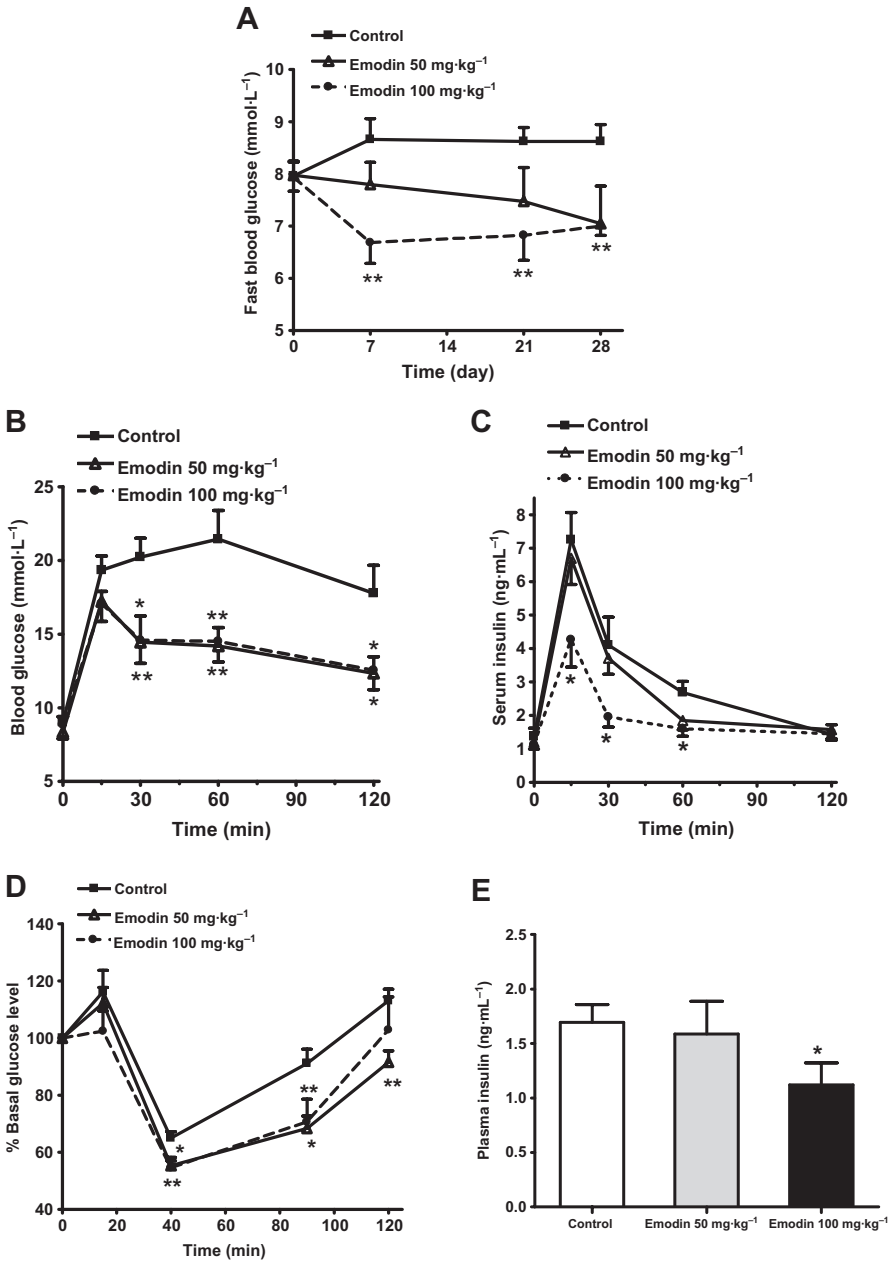

Figure 4

Emodin lowered blood glucose and increased insulin sensitivity of DIO mice. DIO mice were treated as described in Methods. Fasting blood glucose concentrations (A) were measured regularly during the treatment period. Glucose tolerance (B) and the corresponding insulin levels (C) were determined at day 24 of the treatment. Insulin tolerance (D) was determined at day 28 of the treatment. Serum insulin concentration (E) was measured in mice deprived of food for 5 h (5 h fasted mice) at the end of the treatment period. Values are expressed as mean ± SEM for n = 8 mice. *P < 0.05; **P < 0.01 versus control mice.

and is closely associated with the development of a cluster of metabolic abnormalities including insulin resistance, central obesity, hyperglycaemia and dyslipidaemia (Masuzaki *et al.*, 2001; Lindsay *et al.*, 2003; Lundgren *et al.*, 2004; Valsamakis *et al.*, 2004; Abdallah *et al.*, 2005). Thus, there is a great interest in the discovery of potent selective 11β-HSD1 inhibitors for the development of therapeutic interventions in metabolic syndrome. In the present study, a screening of our compound collection provided us with an astonishing discovery that of a series anthraquinone compounds showed inhibitory activities against mouse and human 11β-HSD1. The SPA showed that emodin inhibited mouse and human 11β-HSD1 activity with IC_{50} values of 86 and 186 nM, respectively. As only 79% amino acids of

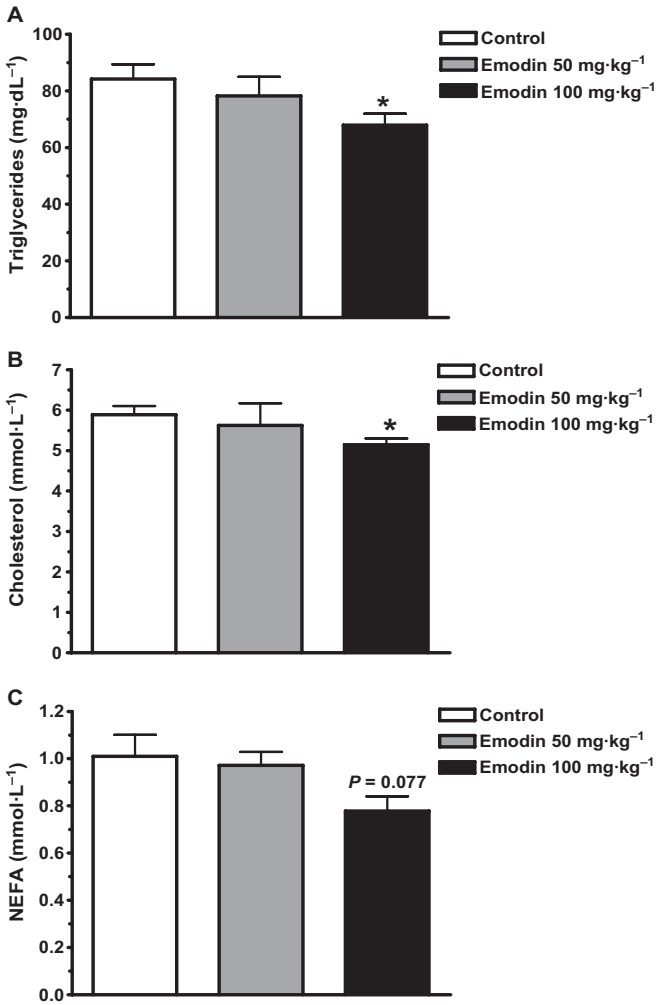

Figure 5

Emodin decreased serum lipids of DIO mice. DIO mice were treated as described in Methods. Serum triacylglycerol (A), cholesterols (B) and NEFA (C) concentrations were evaluated at the end of the treatment period. Data are mean ± SEM for $n = 8$ mice. *$P < 0.05$ versus control mice.

the mouse and human 11β-HSD1 enzymes are identical, we did not expect emodin to inhibit 11β-HSD1 from both species to a similar degree. More importantly, emodin exhibited low inhibitory activity against mouse and human 11β-HSD2, with an IC_{50} higher than 1 mM, indicating that emodin is more than 5000-fold selective for the human and mouse 11β-HSD1 enzymes over the type 2 isoenzyme. A SPA for 11-HSD1 activity was also performed with the liver homogenates, and emodin displayed a comparable IC_{50} value against 11β-HSD1 in cell lysate with the recombinant enzyme (data not shown). Moreover, the *in vivo* inhibitory effect of emodin on 11β-HSD1 was confirmed in C57 BL/6J mice; a significant reduction of 11β-HSD1 activity in liver and mesenteric fat occurred at 2 h post-dose, which is around the half-life time of oral administration of emodin (Li *et al.*, 2009). Therefore, emodin is a potent selective inhibitor of both the *in vitro* and *in vivo* activities of 11β-HSD1.

Chronic exposure to high circulating glucocorticoid levels causes insulin resistance (Pagano *et al.*, 1983). In the present study, chronic treatment of C57BL/6J mice with dexamethasone or prednisone resulted in an impaired insulin tolerance, which indicated the development of insulin resistance. Concurrent treatment with emodin had no effect on dexamethasone-induced insulin resistance, whereas prednisone-induced insulin resistance could be fully reversed by emodin. Dexamethasone is a synthetic cortisol analogue, whereas prednisone is a synthetic cortisone analogue and needs to be catalysed by 11β-HSD1 in the liver to convert it into its active metabolite, prednisolone. Therefore, the finding that emodin prevented prednisone-induced insulin resistance confirmed that chronic administration of emodin can inhibit hepatic 11β-HSD1 activity *in vivo*.

The DIO mice showed moderate obesity, mild hyperglycaemia, dyslipidaemia and insulin resistance after being fed a high-fat diet for 12–15 weeks, which is closely similar to the obesity seen in humans consuming high-fat and energy-rich diets (Surwit *et al.*, 1988). So, this model of obesity has been extensively used to evaluate the pharmacodynamic effects of numerous therapeutic compounds on metabolic syndrome or type 2 diabetes (Hu *et al.*, 2007; Gounarides *et al.*, 2008). Glucocorticoid excess antagonizes the effects of insulin, which decreases glucose uptake in peripheral tissues, increases hepatic glucose production and leads to elevated circulating levels of glucose and insulin resistance (Lambillotte *et al.*, 1997). Selective inhibition of 11β-HSD1 could provide the means to block local activation of glucocorticoids and ameliorate the metabolic disorders (Hermanowski-Vosatka *et al.*, 2005; Taylor *et al.*, 2008). In the present study, emodin administration decreased blood glucose levels in DIO mice, with a parallel decrease in insulin levels. The OGTT results showed that treatment with emodin 100 mg·kg^{-1} resulted in a significant reduction in blood glucose levels, accompanied by a decrease in serum insulin concentrations, which indicates an increase of insulin sensitivity. This was further confirmed by the ITT results. Inhibition of 11β-HSD1 was expected to have a lipid-lowering effect, based on the ability of glucocorticoids to induce lipolysis and produce hepatic lipoprotein (Slavin *et al.*, 1994). Emodin administration significantly reduced serum triglycerides and cholesterol levels in DIO mice, and tended to reduce the NEFA level, although this did

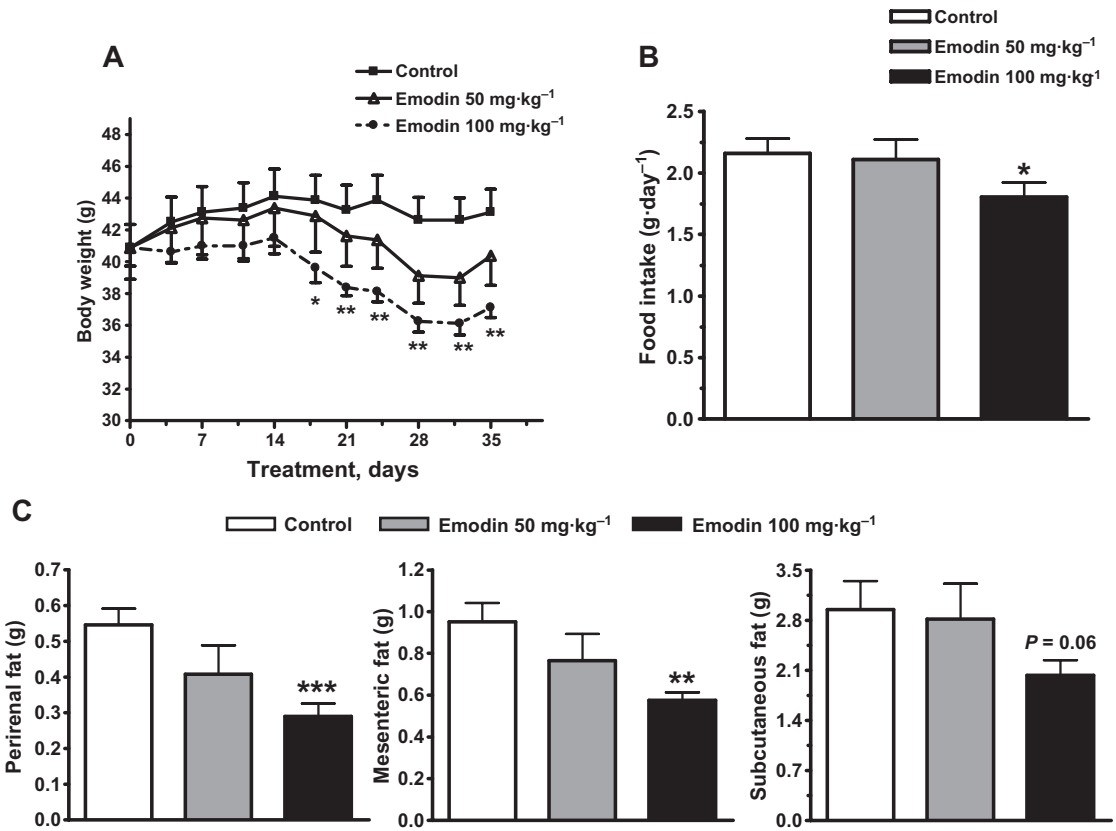

Figure 6

Emodin decreased body weight, food intake and fat pad weight of DIO mice. DIO mice were treated as described in Methods. Body weight (A) and food intake (B) were recorded regularly during the treatment period. Different fat pads (C) were also weighed at the end of the experiment. Values are mean ± SEM for $n = 8$ mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control mice.

not reach statistical significance. This modest decrease in NEFA level may be explained by the 41% inhibition of 11β-HSD1 activity in adipose tissue of emodin-treated mice, which might lead to only a slight suppression of the lipolytic activity induced by active glucocorticoids. Our results are consistent with previous reports on the effects of selective 11β-HSD1 inhibitors and on observations obtained in 11β-HSD1 KO mice (Morton *et al.*, 2001), which suggested that emodin ameliorates metabolic disorder in DIO mice by selective inhibition of 11β-HSD1 in liver and adipose tissues.

Glucocorticoids are orexigenic (Cavagnini *et al.*, 2000), and overexpression of 11β-HSD1 selectively in adipose tissue causes hyperphagia (Masuzaki *et al.*, 2001). A previous study showed that the 11β-HSD1 inhibitor, BVT.2733 reduced food intake and body weight gain, but maintained energy expenditure in DIO mice, although the impared feeding caused a decrease of body weight as great as the inhibitor treatment (Wang *et al.*, 2006). Therefore, we speculated that the decreased body weight caused by 100 mg·kg^{-1} emodin could be partly due to the reduced food intake, and the energy expenditure is likely to be maintained in emodin-treated mice as previously reported (Wang *et al.*, 2006). Excess glucocorticoids enhance hypertrophy and differentiation of adipocytes, leading to central obesity and a redistribution of adipose tissue away from subcutaneous depots and into the visceral compartment (Marin *et al.*, 1992; Vegiopoulos *et al.*, 2007; Morton, 2010). Therefore, it is reasonable to assume administration of emodin, via inhibition of 11β-HSD1 activity, lowers the activity of GCs and this decreases the visceral fat mass, as shown here for the DIO mice.

Glucocorticoids stimulate transcription of hepatic gluconeogenic enzymes and thus play a major role in the enhancement of liver glucose output during starvation or stress (Pilkis and Granner, 1992). Thus, inhibition of 11β-HSD1 offers an effective pharmacological intervention that is likely to yield a sustained reduction of glucocorticoid-inducible hepatic gluconeogenic enzymes. PEPCK and G6Pase catalyse the rate-limiting steps of gluconeogenesis. Transcription of

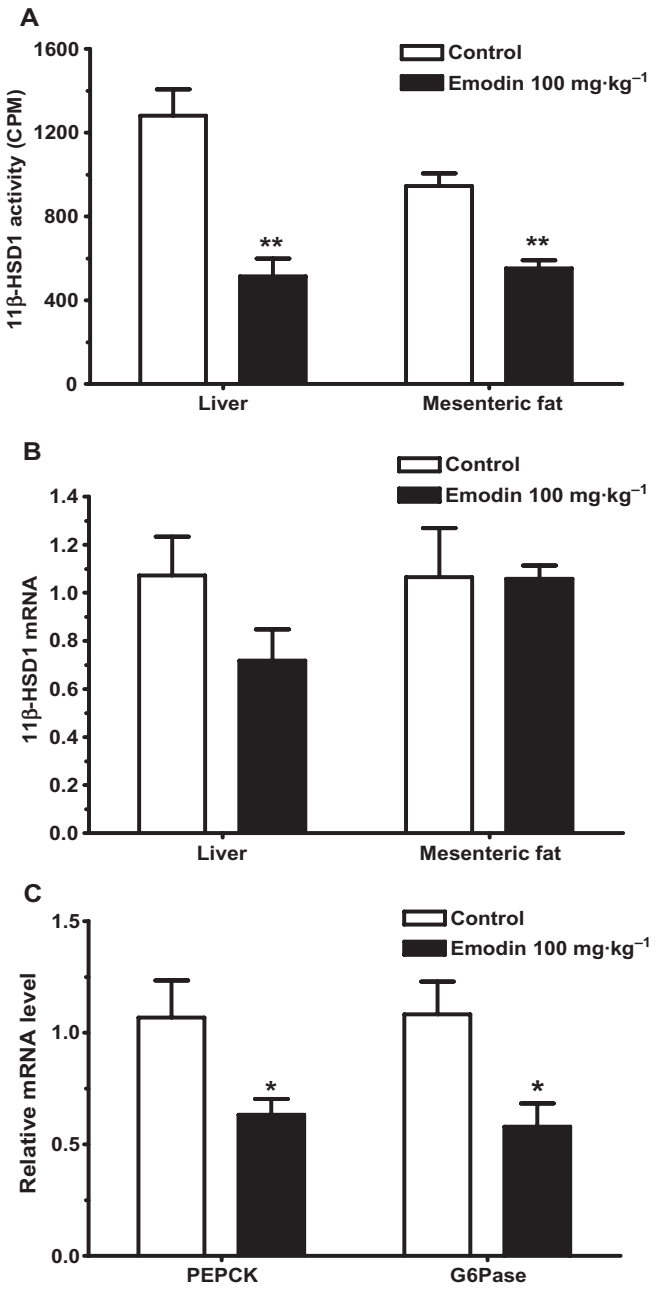

Figure 7

Emodin suppressed 11β-HSD1 activity and reduced the mRNA levels of gluconeogenic genes in DIO mice. DIO mice were treated as described in Methods. 11β-HSD1 activity (A) in liver and mesenteric adipose tissues was measured by SPA at the end of the treatment period. The expression of 11β-HSD1 mRNA (B), PEPCK and G6Pase mRNA (C) were also determined by real-time PCR at the end of the treatment period. Values are mean ± SEM for n = 4–8 mice. *P < 0.05; **P < 0.01 versus control mice.

genes encoding both enzymes is regulated by classical glucocorticoid-inducible promoters (Imai *et al.*, 1990; Lange *et al.*, 1994), and is markedly attenuated in GR-deficient mice (Cole *et al.*, 1995). Administration of emodin significantly reduced hepatic concentrations of mRNA encoding PEPCK and G6Pase, which is consistent with observations in 11β-HSD1 knock-out mice and with the selective inhibitor BVT.2733 (Alberts *et al.*, 2003). These results support the hypothesis that emodin is a potent 11β-HSD1 inhibitor, which can reduce GR-activated hepatic gluconeogenesis; this might account for the decreased fasting blood glucose level and the improvement of the glucose tolerance seen after emodin treatment.

Glycyrrhetinic acid, a natural compound, and its hemisuccinyl derivative carbenoxolone have been well documented as 11β-HSD1 inhibitors (Walker *et al.*, 1995). However, these two compounds display poor selectivity between the two isoforms of 11β-HSDs (Nuotio-Antar *et al.*, 2007). Although, in a clinical study, carbenoxolone has been reported to improve hepatic insulin sensitivity and decrease glucose production in euglycaemic hyperinsulinaemic clamp, it only inhibited 11β-HSD1 in liver but had no effect in adipose tissue *in vivo* (Taylor *et al.*, 2008). In our study, chronic treatment with emodin caused significant inhibition of 11β-HSD1 activity both in liver and mesenteric adipose tissue of DIO mice, whereas the 11β-HSD1 mRNA levels did not tend to change significantly.

Accumulating studies have indicated that a more effective targeting of 11β-HSD1 on adipose tissue is needed (Engeli *et al.*, 2004; Morton *et al.*, 2004; Tomlinson *et al.*, 2007), our data suggest that of all the natural products showing 11β-HSD1 inhibitory activity, emodin is the most selective inhibitor of 11β-HSD1. Moreover, although the affinity of emodin for other enzymes and receptors has not been investigated, no evidence was found that emodin has any significant affinity for a panel of essential and ubiquitous enzymes and receptors, including the oestrogen, glucocorticoid, progesterone and androgen receptors.

In conclusion, our studies demonstrate a new role for emodin as a potent selective inhibitor of 11β-HSD1. Administration of emodin decreased blood glucose and serum insulin, improved insulin resistance and dyslipidaemia and decreased body weight and central fat mass in DIO mice. These results highlight the potential value of analogues of emodin as a new class of compound for the treatment of metabolic syndrome or type 2 diabetes.

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Conflict of interest

None of the authors have any conflict of interest.

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