ORIGINAL ARTICLE

Effective treatment with combination of peripheral 5-hydroxytryptamine synthetic inhibitor and 5-hydroxytryptamine 2 receptor antagonist on glucocorticoid-induced whole-body insulin resistance with hyperglycemia

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ABSTRACT

Aims/Introduction: Our previous study found that dexamethasone-induced insulin resistance (IR) was involved in 5-hydroxytryptamine (5-HT) synthesis and 5-hydroxytryptamine 2 receptor (5-HT$_2$R) in the periphery. The present study examined the effects of inhibitions of both peripheral 5-HT synthesis and 5-HT$_2$R on dexamethasone-induced IR.

Materials and Methods: Male rats were exposed to dexamethasone for 10 days, then treated with or without a 5-HT$_2$R antagonist, sarpogrelate, a 5-HT synthetic inhibitor, carbidopa, alone or in combination for 20 days.

Results: Dexamethasone-induced whole-body IR, with glucose intolerance, decreased insulin sensitivity, hyperglycemia, hyperinsulinemia and dyslipidemia, could be effectively abolished by sarpogrelate or/and carbidopa, whereas IR-related actions of dexamethasone in tissues were accompanied by increased 5-HT synthesis in the liver and visceral adipose, and upregulated 5-HT$_2$R (5-HT$_{2A}$R and 5-HT$_{2B}$R) expression in these two tissues as well as in skeletal muscle. Sarpogrelate or/and carbidopa treatment significantly abolished dexamethasone-caused tissue-specific IR. In the liver, increased gluconeogenesis, triglycerides and very low-density lipoprotein syntheses with steatosis, and downregulated expression of plasmalemmal glucose transporter-2 were markedly reversed. In the visceral adipose and skeletal muscle, downregulated expression of plasmalemmal glucose transporter-4 was significantly reversed, and increased lipolysis was also reversed in the visceral adipose. Dexamethasone-induced activations of hepatic mammalian target of rapamycin serine^{2448}, and S6K threonine$^{389/412}$ phosphorylation were also abolished markedly by sarpogrelate or/and carbidopa. Co-treatment with sarpogrelate and carbidopa showed a synergistic effect on suppressing dexamethasone actions.

Conclusion: Inhibitions of both peripheral 5-HT synthesis and 5-HT$_2$R are expected to be a dependable target for treatment of steroid-induced diabetes.

INTRODUCTION

Glucocorticoids (GCs) are frequently prescribed anti-inflammatory and immunosuppressive drugs, but they have an extensive side-effect profile, such as development of type 2 diabetes, ischemic heart diseases, dyslipidemia, osteoarthritis, depression and especially whole-body insulin resistance (IR)[1]. Chronically elevated GC levels have been linked to fatty liver development, and as a result, could contribute to hepatic steatosis[2]. Insulin-resistant and glucose-intolerant patients have an elevated GC

level[3,4], and GC is also associated with the fatty liver phenotype in non-alcoholic fatty liver disease[5], myotonic dystrophy[6] and metabolic obesity in normal-weight subjects[7]. The liver is an important player in the diabetogenic effects induced by GC treatment[8], whereas pathophysiological accumulation of lipids in the liver has been identified as an independent risk factor for IR and metabolic syndrome[9]. In addition, GC is also involved in IR in adipose tissue and skeletal muscle[1].

Serotonin, also called 5-hydroxytryptamine (5-HT), is synthesized by a two-step enzymatic pathway, in which tryptophan is first converted to 5-hydroxy-tryptophan (5-HTP) by the enzyme tryptophan hydroxylase (Tph), and 5-HTP is next converted to 5-HT by aromatic L-amino acid decarboxylase (AADC)[10,11]. There are two subtypes of Tph, Tph1 and Tph2, presenting in the periphery (Tph1) and center (Tph2), respectively[12]. As to 5-HT receptors (5-HTR), seven receptor classes, including 14 subtypes of 5-HTR belonging to seven subfamilies ($5-HT_1R$ to $5-HT_7R$), have been identified to date, reflecting the diversity of serotoninergic actions[13]. The $5-HT_2R$ family including three subtypes, named $5-HT_{2A, 2B, 2C}$ receptors, are expressed predominantly in peripheral tissues, with similar structure, pharmacological properties and signaling pathways[13]. It has been found that serum levels of 5-HT are elevated in diabetic patients, and the increasing concentration in serum is a marker of diabetic complications[14–16]. Therefore, glucose metabolism and obesity might be etiologically associated with 5-HT[17,18], as well as adipocyte differentiation mediated by $5-HT_2Rs$ *in vitro*[19]. Upregulation of the $5-HT_{2A}R$ is often found in obesity and diabetes, which leads to high blood sugar level[20], and GC treatment can increase density of serotonin $5-HT_{2A}R$ in humans[21].

In a previous study[22], we found that 5-HT is synthesized in both the liver and visceral adipose tissues, which are enhanced by chronic GC exposure, and is important for GC-induced IR in both organs and whole body; GC also upregulates expressions of $5-HT_{2A}R$ and $5-HT_{2B}R$ in both organs, which might be another reason for GC-induced IR. To examine the hypothesis that GC-induced IR can be effectively treated by inhibition of both peripheral tissue's 5-HT synthesis and $5-HT_2R$, insulin-resistant rats induced by long-term exposure to dexamethasone (Dex) were treated with an AADC inhibitor, carbidopa (CDP), or/and a $5-HT_2R$ antagonist, sarpogrelate (Sar). The parameters related with whole-body IR, and IR-related abnormality in the liver, visceral adipose and skeletal muscle tissue were examined.

MATERIALS AND METHODS
Animal experiments
All studies were carried out in accordance with the Laboratory Animal Care Committee at China Pharmaceutical University. Animals were kept on a standard 12-h light/dark cycle with access to water and food *ad libitum* throughout the experiment. First, male Sprague–Dawley rats (10-weeks-old, purchased from B&K Universal Group Limited Shanghai, China; license number: SCXK [Hu] 2013-0006) were subcutaneously given normal saline (control rats) or 0.75 mg/kg bodyweight Dex

(Dexamethasone Sodium Phosphate Injection; Cisen Pharmaceutical Co., Ltd, Jining, China; diluted with normal saline) twice daily on the morning and afternoon with a 12-h interval for 10 days, to make a model of Dex-induced IR. We found that long-term treatment with 2.0 mg/kg bodyweight Dex twice daily, as had been carried out in another investigation[23], easily led to increased mortality of rats, whereas a dose of 0.75 mg/kg bodyweight twice daily was safer, and also induced a marked IR in these rats. The consequences of hyperglycemia and hyperinsulinemia were judged by measuring the levels of fasting blood glucose and blood insulin on day 10 after initiating Dex exposure. Then, the Dex-exposed rats were divided into four groups randomly (*n* = 8 per group): model group, Dex-exposed with Sar (sarpogrelate hydrochloride; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan), a broad-spectrum antagonist of 5-HT$_2$R, -treated group (Sar group), Dex-exposed with CDP (Sigma, St. Louis, MO, USA), an inhibitor of AADC, -treated group (CDP group), and Dex-exposed with Sar and CDP co-treated group (Sar+CDP group). The treatments were twice-daily, carried out for 20 days with an oral administration at 1 h before Dex exposure. In the Sar group, Sar at 25 mg/kg bodyweight was given twice daily before Dex exposure, which was lower than previously reported[24]. In order to execute a parallel comparison between Sar and CDP treatment, 25 mg/kg bodyweight CDP treatment twice daily was also carried out in the CDP group, whereas that in the Sar+CDP group was of a mixture with both (Sar : CDP = 2:1) as an equal dose with both the Sar and CDP group. The drugs were all dissolved with a vehicle 0.5% CMC-Na, and were made to the same concentration of 5.0 mg/mL with the same delivery volume (0.50 mL/kg bodyweight), whereas rats in the control and model group were given 0.5% CMC-Na (0.50 mL/kg bodyweight).

On day 16 and day 18 of treatment, the glucose tolerance test (GTT) and insulin tolerance test (ITT) were carried out at 12 h after fasting, and 5 h after the drug and Dex treatment. At the end of the experiment, animals were deprived of food (free to take water) for 12 h, and then were anesthetized by amobarbital sodium (45 mg/kg) intraperitoneal injection and euthanized. Collected blood samples were centrifuged (600 *g*, for 10 min) for obtaining serum. Liver tissue, intra-abdominal adipose tissue, including mesenteric, bilateral perirenal and epididymal adipose tissue, and hind thigh muscle were removed immediately. Liver tissue and intra-abdominal adipose tissue were weighed. The tissues were washed in cold phosphate-buffered saline. Samples of serum and the tissues were stored at −80°C immediately for subsequent further measurement.

GTT and ITT
Randomly selected five of eight rats in each group were used to carry out GTT and ITT. A dose of 2 g/kg glucose injection (Hunan Kelun Pharmaceutical Co., Ltd, Yueyang, China) was intraperitoneally injected. Approximately 20 μL of blood was sampled at 0, 30, 60, 90 and 150 min by tail bleeding before

and after glucose was given. Blood glucose was measured using a LifeScan Blood Glucose Meter (Johnson & Johnson, Milpitas, CA, USA). The GTT was evaluated by the total area under the blood glucose curve (AUC) using the trapezoidal method[25].

A dose of 0.5 IU/kg insulin injection (Eli Lilly Inc., Suzhou, China) was intraperitoneally injected. Approximately 20 μL of blood was sampled at 0, 30, 60, 90 and 120 min by tail bleeding before and after insulin was given. Blood glucose was measured, and the ITT was evaluated by AUC like GTT.

Serum and hepatic biochemical analysis

The 0.4-g tissues were sliced and homogenized in 4 mL cold phosphate-buffered saline buffer, and the homogenate was then used for measurement. Levels of 5-HT, insulin, dopamine and very low-density lipoprotein (VLDL) in the tissue or serum were measured by using an enzyme-linked immunosorbent assay kit (Abcam, Sha Tin, Hong Kong). Serum levels of triglyceride (TG), total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), free fatty acids (FFA) and glucose, and glycerol content in the adipose tissue were measured by using a spectrophotometer kit (Nanjing Jianchen, Nanjing, China). TG content in the liver tissue was measured using a TG enzyme-test kit (Applygen Technologies Inc., Beijing, China). Serum VLDL-c level was calculated by VLDL-c = total cholesterol – LDL-c – HDL-c. Homeostasis model of assessment for IR index was calculated by serum glucose × serum insulin / 22.5[26].

Oil red O staining in the liver tissue

The liver tissue stored in a −80°C freezer was placed in an optimal cutting temperature chamber and a 6-μm thick section was made. Tissue slices were rinsed with phosphate-buffered saline, raised with 60% isopropanol and stained with fresh Oil Red O working solution for 15 min. Tissue slices were rinsed with 60% isopropanol again, and hematoxylin was used to counterstain for showing the nucleus.

Reverse transcription polymerase chain reaction

Total ribonucleic acid (RNA) was extracted from intra-abdominal adipose tissue or liver tissue using RNAiso Plus Isolation Reagent (TAKARA, Otsu, Shiga, Japan) according to the manufacturer's instructions. Total RNA was reserve transcribed and amplified in a GeneAmp PCR system (Eppendorf, Hamburg, Germany). Primers used in the reverse transcription polymerase chain reaction were of: adipose triglyceride lipase (ATGL; forward TTC AAG TTT CCT TGC AGA GT; reverse CTC CCA AAC TGA CCC TTA AA) in visceral adipose tissue, acetyl-CoA carboxylase (ACCase; forward GCC AGC AGA ATT TGT TAC TC; reverse AGA CGA TGC AAT CTT ATC CC) in liver tissue, and glyceraldehyde 3-phosphate dehydrogenase (forward TAT CGG ACG CCT GGT TAC; reverse TGC TGA CAA TCT TGA GGG A). Data analysis was carried out using a GeneGenius automatic gel imaging and analysis system (Syngene, Cambridge, UK), and scanned by densitometry for quantitation. To exclude variations as a result of RNA quantity and quality, the data for genes were adjusted to glyceraldehyde 3-phosphate dehydrogenase, and the relative expression levels of ATGL and ACCase were calculated as: (relative gray value of the gene / mean of relative gray value in the control) × 100%.

Western blotting

Liver tissue, intra-abdominal adipose tissue or skeletal muscle tissue were homogenized in lysis buffer, then sonicated and incubated on ice for 15 min. Extraction of cytosol or membrane protein used a Cytosol or Membrane Protein Extraction Kit (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred onto nitrocellulose membrane. After being blocked, the membranes were then incubated with appropriate primary antibodies, including antibodies with anti-Tph1 and anti-AADC (Epitomics-Abcam, Sha Tin, Hong Kong), anti-glycerin-3-phosphate acyltransferase 1 and microsomal triglyceride transfer protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-glucose transporter 2 (GLUT2) and GLUT4 (Epitomics-Abcam), anti-5-HTR$_{2A}$R and 5-HT$_{2B}$R (GeneTex Inc., Irvine, CA, USA), anti-phosphoenolpyruvate carboxykinase-1, anti-serine (Ser)[2448] phospho-mTOR, anti-mTOR, anti-threonine (Thr)[389/412] phospho-p70S6K and anti-p70S6K (Signalway Antibody, College Park, MD, USA), and anti-β-actin (Bioworld Technology Inc., St. Louis Park, MO, China). Then the membranes were incubated with the HRP-coupled secondary antibodies (HuaAn Biotechnology Co. Ltd, Hangzhou, China). Detection was carried out by densitometry using the enhanced chemiluminescence detection system (Tanon-5200; Tanon Science & Technology Co., Ltd, Shanghai, China). The relative expressions levels of each protein were calculated as: (relative gray value of each protein / mean of relative gray value in the control) × 100%.

Statistical analysis

Data are shown as mean ± standard deviation. The differences among the groups were evaluated by one-way analysis of variance (ANOVA) followed by Fisher's least significant differences tests under the homogeneity of variances or Tamhane's T2 tests under the non-homogeneity of variances, whereas the differences of tissue protein expression levels of Tph1, AADC, 5-H$_{2A}$R, and 5-HT$_{2B}$R between the control and Dex-exposed group were tested by using Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Effects of Sar or/and CDP treatment on Dex-induced whole-body IR and decrease in bodyweight and food intake

Bodyweight in the rats exposed to Dex for 10 days was significantly decreased compared with the control rats, whereas subsequent treatment with Sar or/and CDP for 20 days significantly

Figure 1 | Effects of sarpogrelate (Sar) or/and carbidopa (CDP) treatment on dexamethasone (Dex)-induced alterations in bodyweight, food intake and whole-body insulin resistance in the rats. (a) Bodyweight (left) and food intake (right, only mean value), (b) glucose tolerance test with blood glucose levels (left) and area under the blood glucose curve (AUC; right), and (c) insulin tolerance test with blood glucose levels (left) and (c) AUC (right) were shown in the control (Ctrl), model with Dex-exposed (Dex), and Dex-exposed with Sar (Dex+Sar), CDP (Dex+CDP), or Sar and CDP (Dex+Sar+CDP)-treated group. (d) Levels of serum glucose (left) and insulin (middle), homeostasis model of assessment for insulin resistance (HOMA-IR) index (right), and (e) levels of serum TG (left), low-density lipoprotein cholesterol (LDL-c) and very low-density lipoprotein cholesterol (VLDL-c; middle), and high-density lipoprotein cholesterol (HDL-c; right) are shown in each group. Data are presented as the mean ± standard deviation. Except for five of eight per group in the glucose and insulin tolerance tests, the others were from all eight rats per group. (b,c) *$P < 0.05$, **$P < 0.01$ in the blood glucose compared the model group with each group, or in other data.

reversed Dex-caused bodyweight loss with a significant bodyweight gain compared with the Dex-exposed rats. In addition, the bodyweight between in Sar- or/and CDP-treated groups was not different, which was also not different compared with that before drug treatment in each group (Figure 1a, left), showing that the Sar or/and CDP treatment completely suppressed Dex-caused weight loss. Food intake was also decreased by Dex, which was not reversed significantly by Sar or/and CDP treatment, with a slight attenuation by the drug treatment, especially in the CDP and Sar+CDP groups (Figure 1a, right).

Fasting blood glucose level (Figure 1b, left) at the beginning of GTT was minimally but significantly, increased in the Dex-exposed rats (model group) compared with the control rats, and was lower in the Sar, CDP and Sar+CDP groups than in the model group. A more glucose-stimulated increase in blood glucose level (Figure 1b, left) within 150 min in the Dex-exposed rats was found compared with the control, and AUC (Figure 1b, right), a marker of glucose intolerance, was markedly elevated by Dex, both of which were significantly suppressed by Sar or CDP treatment, and were more effectively suppressed by both co-treatment, suggesting a synergistic effect between Sar and CDP. We also detected a Dex-induced impairment of insulin tolerance examined by ITT, in which both insulin-stimulated decrease in blood glucose level (Figure 1c, left) within 120 min and AUC (Figure 1c, right) in the Dex-exposed rats were blunted compared with the control, whereas both were markedly improved by Sar or/and CDP treatment with a synergistic effect between Sar and CDP.

In the end of the experiment, hyperglycemia (Figure 1d, left) and hyperinsulinemia (Figure 1d, middle) with an increased homeostasis model of assessment for IR index (Figure 1d, right) induced by Dex were found, which were attenuated significantly by Sar or/and CDP treatment with a synergistic effect between them. Dyslipidemia with increased levels of serum TG (Figure 1e, left), LDL-c and VLDL-c (Figure 1e, middle), and decreased serum level of HDL-c (Figure 1e, right) was also detected in the Dex-exposed rats, which was also attenuated by Sar or/and CDP treatment with a synergistic effect between them. More importantly, higher serum HDL-c level in the Sar+CDP group than the control group were detected. The results suggested that Dex-induced whole-body IR could be markedly improved by Sar or CDP treatment, whereas co-treatment with Sar and CDP has a strong abolishment with a synergistic effect on Dex-induced IR.

Effects of Sar or/and CDP on Dex-induced energy metabolic disorder in the liver

Liver weight (Figure 2a, left up) was significantly decreased in the model group compared with the control, whereas the liver to bodyweight ratio, namely hepatic index (Figure 2a, left down), was markedly increased. Either Sar or CDP treatment showed a reversed tendency on Dex-induced decrease in liver weight and increase in hepatic index, whereas the co-treatment markedly reversed Dex effect on hepatic index, and had a reversed tendency on liver weight. Significant hepatic steatosis (Figure 2a, right) examined by using Oil Red O staining was found in the model group, which was inhibited by Sar or CDP treatment, and was strongly inhibited by the co-treatment, indicating a synergistic effect between both.

TG synthesis detected by assessing glycerin-3-phosphate acyltransferase 1 expression (Figure 2b, left), a rate-limiting enzyme of hepatic TG synthesis[27] and VLDL assembly detected by assessing microsomal triglyceride transfer protein expression (Figure 2b, left), a key factor of hepatic VLDL assembly[28], both were upregulated by Dex. Sar or CDP treatment obviously suppressed Dex-induced upregulations of glycerin-3-phosphate acyltransferase 1 and microsomal triglyceride transfer protein, whereas the co-treatment showed strong suppression on Dex effects. Accordingly, increased content of TG (Figure 2b, right upper) and VLDL (Figure 2b, right lower) in the liver tissue of the model group were detected, both of which were significantly decreased by Sar or CDP treatment and strongly decreased by the co-treatment. By assessing hepatic ACCase gene expression (Figure 2b, middle), a rate-limiting enzyme of fatty acids (FAs) synthesis[29], we also detected increased hepatic FAs synthesis with markedly upregulated expression of the ACCase gene in the liver of the model group, which was markedly inhibited by Sar or CDP treatment, but was not further inhibited by the co-treatment. These results suggested that Dex-stimulated TG and VLDL syntheses, showing increased hepatic TG and VLDL content, and steatosis, could be abolished effectively by Sar or CDP treatment and strongly abolished by the combination of both. Sar or CDP treatment also attenuated the Dex-induced FAs synthesis in the liver, but did not have a synergistic effect.

Phosphoenolpyruvate carboxykinase-1 (Figure 2c), a rate-limiting enzyme of gluconeogenesis[1], was upregulated by Dex, whereas GLUT2 expression (Figure 2c) on the cell membrane was downregulated by Dex, and both were significantly
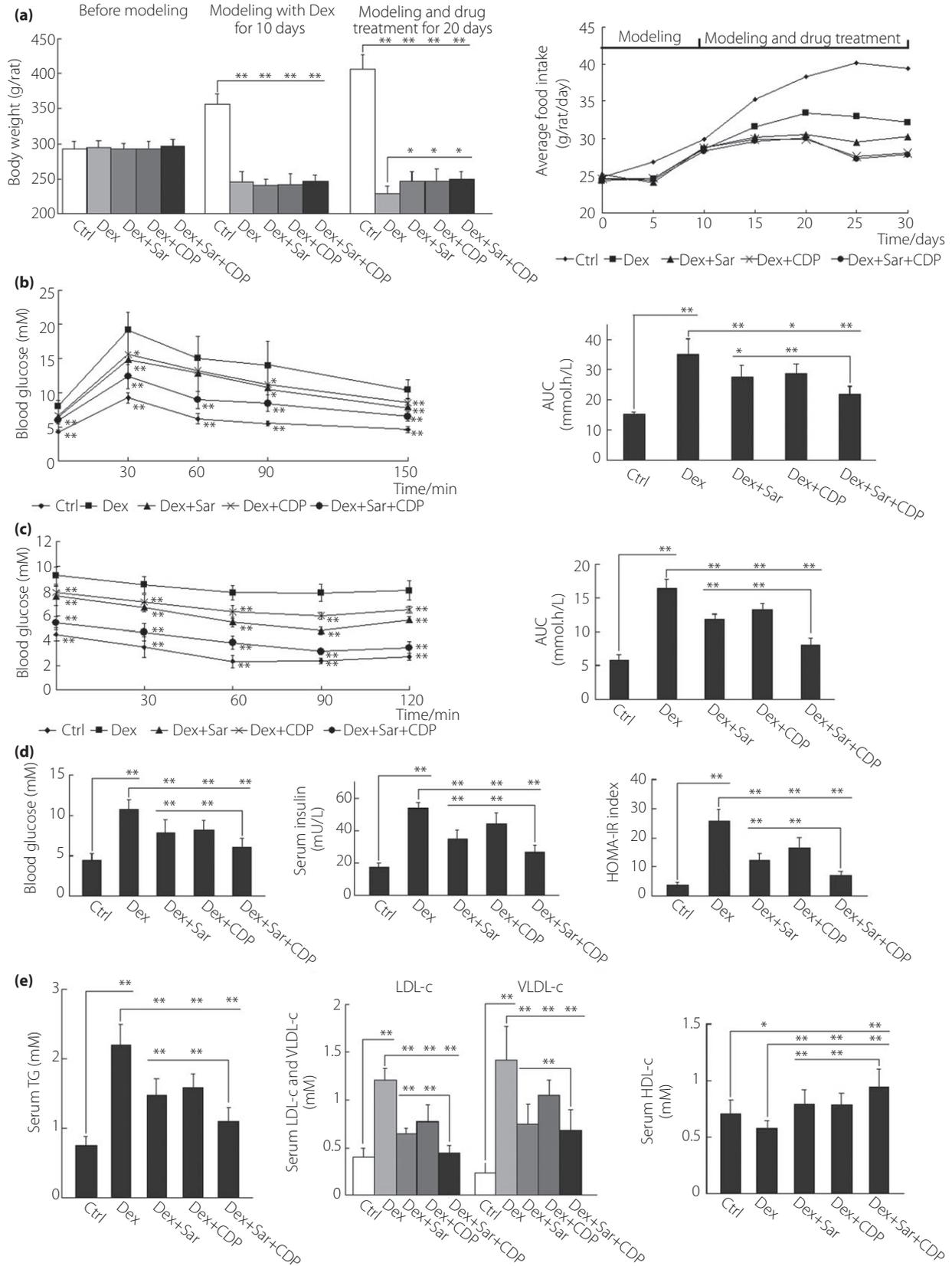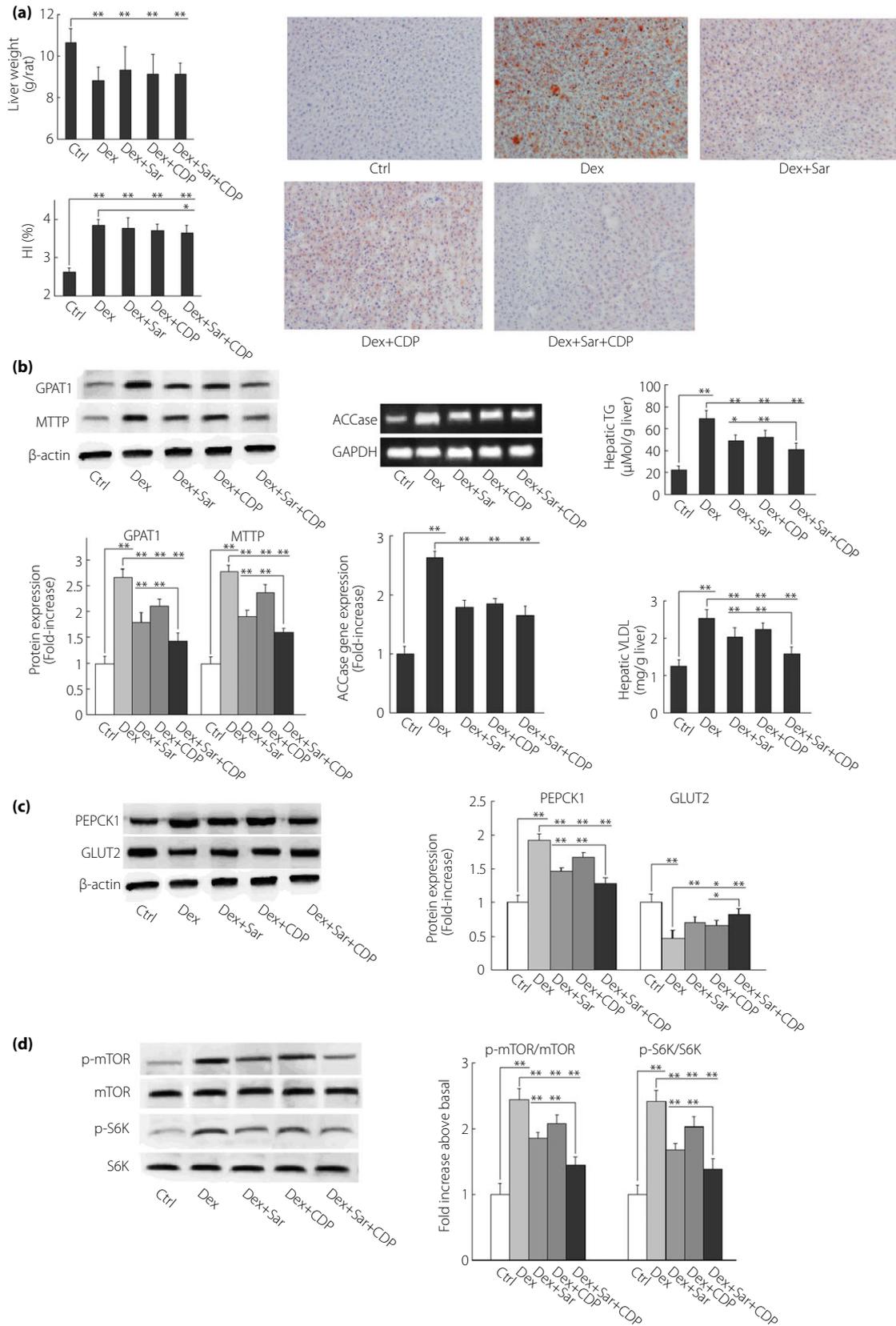
Figure 2 | Effects of sarpogrelate (Sar) or/and carbidopa (CDP) treatment on dexamethasone (Dex)-induced alterations in hepatic lipid and glucose metabolism in the rats. (a) Liver weight (left upper), hepatic index (HI) (left lower) and hepatic steatosis with a representative image (magnification: ×100) using Oil-red O staining (right). (b) Expressions of glycerin-3-phosphate acyltransferase 1 (GPAT1) and microsomal triglyceride transfer protein (MTTP) (left), messenger ribonucleic acid expression of acetyl-CoA carboxylase (ACCase; middle), hepatic contents of triglyceride (TG; right upper) and very low-density lipoprotein cholesterol (VLDL; right lower). (c) expressions of phosphoenolpyruvate carboxykinase-1 (PEPCK1) and glucose transporter 2 (GLUT2), and (d) expressions of serine[2448] phospho-mammalian target of rapamycin (p-mTOR), mTOR, threonine[389/412] phospho-p70S6K(p-S6K), and p70S6K (S6K) are shown in the liver tissues of the control (Ctrl), model with Dex-exposed (Dex) and Dex-exposed with Sar (Dex+Sar), CDP (Dex+CDP), or Sar and CDP (Dex+Sar+CDP)-treated groups. Data are presented as the mean ± standard deviation. Except for four of eight per group in the examining protein or gene expression, the others were from all eight rats per group. *$P < 0.05$, **$P < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

reversed by Sar or CDP treatment and strongly reversed by the co-treatment, showing that the combination of Sar and CDP also had a synergistic effect on abolishing Dex-stimulated increase in gluconeogenesis and decrease in glucose uptake in the liver.

Activations of mTOR Ser[2448] and S6K Thr[389/412] phosphorylation are very important in inducing IR with the regulation of lipogenesis in liver[30,31]. We found that the expressions of mTOR and S6K in the liver (Figure 2d) were not obviously changed by Dex exposure or Dex exposure with Sar or/and CDP treatment, whereas Ser[2448] phosphorylation of mTOR, and Thr[389/412] phosphorylation of p70S6K (Figure 2d) were upregulated markedly by Dex exposure, both of which were abolished significantly by Sar or CDP treatment, and abolished further by the co-treatment, showing a synergistic effect between Sar and CDP. These results suggested that Sar or/and CDP's effect on Dex-induced IR in the liver is closely involved in suppressing Dex-induced activation of the mTOR-S6K pathway.

Effects of Sar or/and CDP on Dex-induced decrease in plasmalemmal GLUT4 expression in the skeletal muscle and visceral adipose tissue, and lipolysis in the visceral adipose tissue

GLUT4 is the most important transporter of glucose from extracellular into intracellular sites in the muscle and adipose tissue, which is translocated by insulin stimulation from intracellular sites to the plasma membrane[32]. Dex-induced downregulation of GLUT4 expression on the cell membrane in the skeletal muscle and visceral adipose tissues were detected, which was reversed by Sar or CDP treatment, and more effectively reversed by the combination of Sar and CDP (Figure 3a, left), showing a synergistic effect of them on abolishing Dex-caused decrease in glucose uptake.

GC promotes lipolysis in the adipose tissue[33]. ATGL is a key enzyme in controlling lipolysis, which can be upregulated by GC[1]. We detected Dex-stimulated lipolysis in the visceral adipose tissue of the model group, with increased serum FFA levels (Figure 3a, right), decreased visceral adipose weight (Figure 3b, left), upregulated expression of the *ATGL* gene (Figure 3b, middle) and increased glycerol content (Figure 3b, right) in the visceral adipose tissue. Co-treatment with Sar and CDP showed more significant amelioration than alone on Dex-

induced lipolysis in visceral adipose tissue (Figure 3b). The co-treatment was more effective than CDP alone on decreasing Dex-caused enhancement in the serum FFA level, ATGL expression and glycerol content, which was also more effective than Sar on reversing glycerol increase. In addition, either Sar or CDP treatment merely showed a reversing tendency on Dex-caused decrease in visceral adipose weight, whereas the co-treatment showed significant reversion on that (Figure 3b).

5-HT Synthesis was upregulated by Dex in the liver and visceral adipose tissue, whereas expressions of 5-HT$_{2A}$ and 5-HT$_{2B}$ were also upregulated by Dex in the liver, visceral adipose and skeletal muscle tissue

To examine whether 5-HT is synthesized and is influenced by Dex in the liver, visceral adipose and skeletal muscle tissue of rats, we assessed Tph1 and AADC expression by western blot, and 5-HT levels in these three tissues and serum. In agreement with a previous study[22], the expressions of Tph1 and AADC (Figure 4a,b) were detected in the liver and visceral adipose tissues, but were not detected in the skeletal muscle (data not shown), whereas rats' chronic exposure to Dex showed markedly upregulated Tph1 and AADC expression in both tissues. Accordingly, 5-HT levels in the serum and three tissues (Figure 4d) were increased significantly by Dex, which were inhibited by CDP in a dose-dependent manner in the CDP or Sar and CDP co-treatment rats, whereas Sar alone did not obviously change 5-HT levels in the serum or in the three tissues. More importantly, the fold increases of 5-HT levels in the liver and visceral adipose tissue in the model group were higher than that in the serum, whereas that in the serum and skeletal muscle tissue were similar, showing that increased 5-HT by Dex in the liver and visceral adipose tissue come from 5-HT synthesis in the tissue itself rather than serum, which was different with 5-HT inside the skeletal muscle, which comes from the serum. Upregulated expressions of 5-HT$_{2A}$R and 5-HT$_{2B}$R by Dex in the three tissues were also detected (Figure 4a–c). In addition, we found very low content of dopamine in the liver, skeletal muscle and visceral adipose tissue, which was not altered by Dex exposure with or without CDP or/and Sar treatment (data not shown), suggesting that Dex-induced IR and CDP or/and Sar effects in the liver, skeletal muscle and visceral adipose tissue were not associated with dopamine.
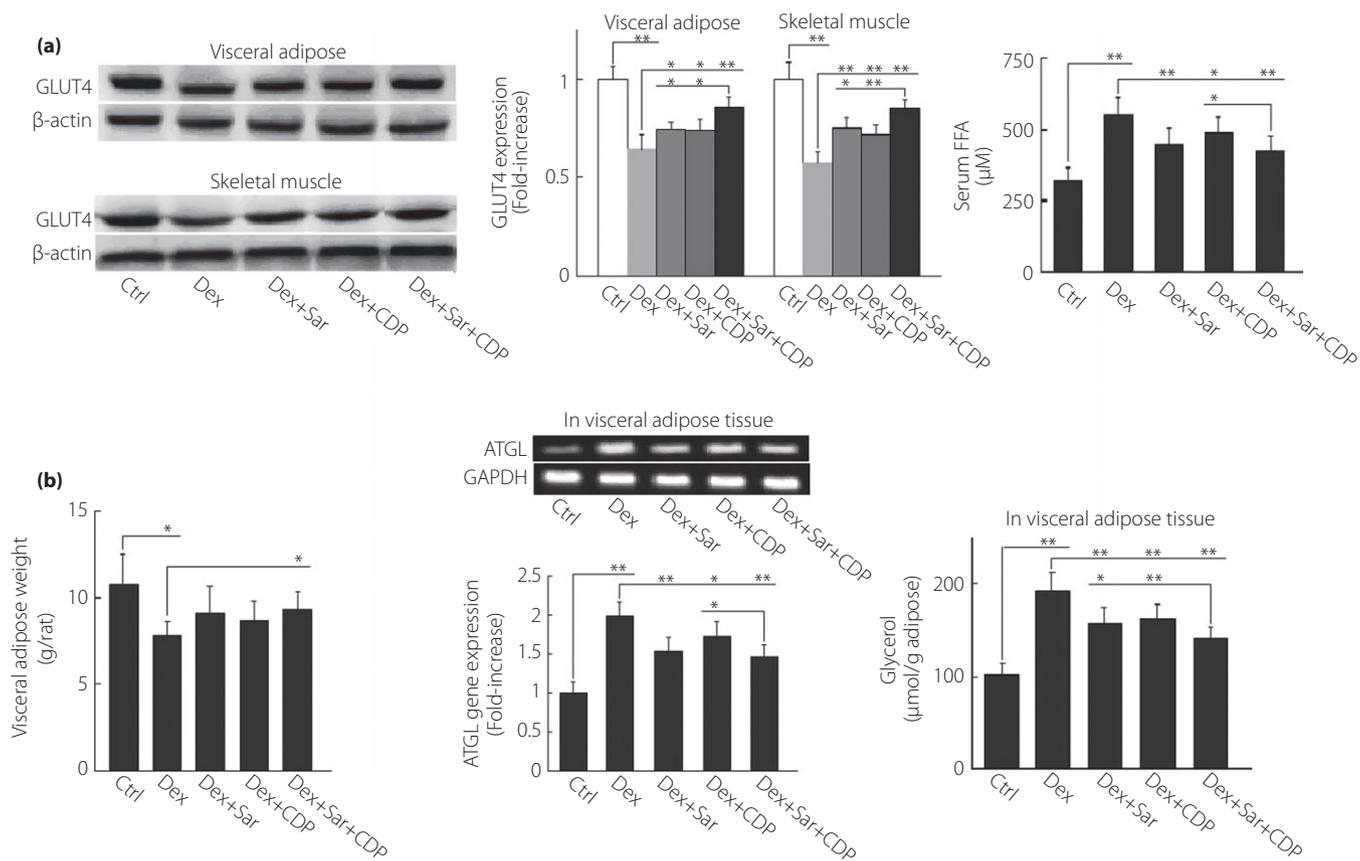
Figure 3 | Effects of sarpogrelate (Sar) or/and carbidopa (CDP) treatment on dexamethasone (Dex)-induced alterations in skeletal muscle and visceral adipose glucose uptake and visceral adipose lipolysis in the rats. Expression of (a) plasmalemmal glucose transporter 4 (GLUT4) in the visceral adipose and skeletal muscle tissue (left), serum free-fatty acids (FFA) level (right), (b) visceral adipose weight (left), and messenger ribonucleic acid expression of adipose triglyceride lipase (ATGL; middle) and glycerol content (right) in the visceral adipose tissue are shown in the control (Ctrl), model with Dex-exposed (Dex), and Dex-exposed with Sar (Dex+Sar), CDP (Dex+CDP), or Sar and CDP (Dex+Sar+CDP)-treated groups. Data are presented as the mean ± standard deviation. Except for four of eight per group in examining GLUT4 and ATGL expression, the others were from all eight rats per group. *$P < 0.05$, **$P < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

These results suggested that Dex-induced upregulations of 5-HT$_2$R and 5-HT synthesis in the liver and visceral adipose tissues, and upregulation of 5-HT$_2$R in the skeletal muscle are very likely important for Dex-caused whole-body IR with tissue-specific IR.

DISCUSSION

GC-induced whole-body IR involves the liver, adipose tissue and skeletal muscle. In the liver, GC stimulates hepatic gluconeogenesis through induction of phosphoenolpyruvate carboxykinase-1 and glucose-6-phosphatase[1], enhances insulin-stimulated hepatic *de novo* lipogenesis by upregulation of ACCase and FA synthase[34,35], and increases VLDL production and secretion[36]. Several indirect mechanisms also likely play a role in GC-induced hepatic lipid accumulation, including increased lipolysis in visceral adipose tissue, which results in more FFA to be delivered to the liver through the blood, and systemic hyperinsulinemia and hyperglycemia, which drive hepatic *de novo* lipogenesis[37]. Ectopic lipid accumulation in the liver have been strongly associated with hepatic IR with whole-

Figure 4 | Examinations of tryptophan hydroxylase 1 (Tph1), aromatic L-amino acid decarboxylase (AADC), 5-hydroxytryptamine 2A receptor (5-HT$_{2A}$R) and 5-HT2B receptor (5-HT$_{2B}$R) expression. Expressions of Tph1, AADC, 5-HT$_{2A}$R and 5-HT$_{2B}$R in the (a) liver and (b) visceral adipose tissue, expressions of (c) 5-HT$_{2A}$R and 5-HT$_{2B}$R in the skeletal muscle tissue were shown in the control (Ctrl) and model with Dex-exposed (Dex) groups. (d) Fold-increases of 5-HT levels in the serum, tissues of liver, visceral adipose, and skeletal muscle were shown in the Ctrl, Dex and Dex-exposed with Sar (Dex+Sar), CDP (Dex+CDP), or Sar and CDP (Dex+Sar+CDP)-treated groups. Data are presented as the mean ± standard deviation. Except for eight per group in examining 5-HT levels, the others were from four of eight rats per group. *$P < 0.05$, **$P < 0.01$.
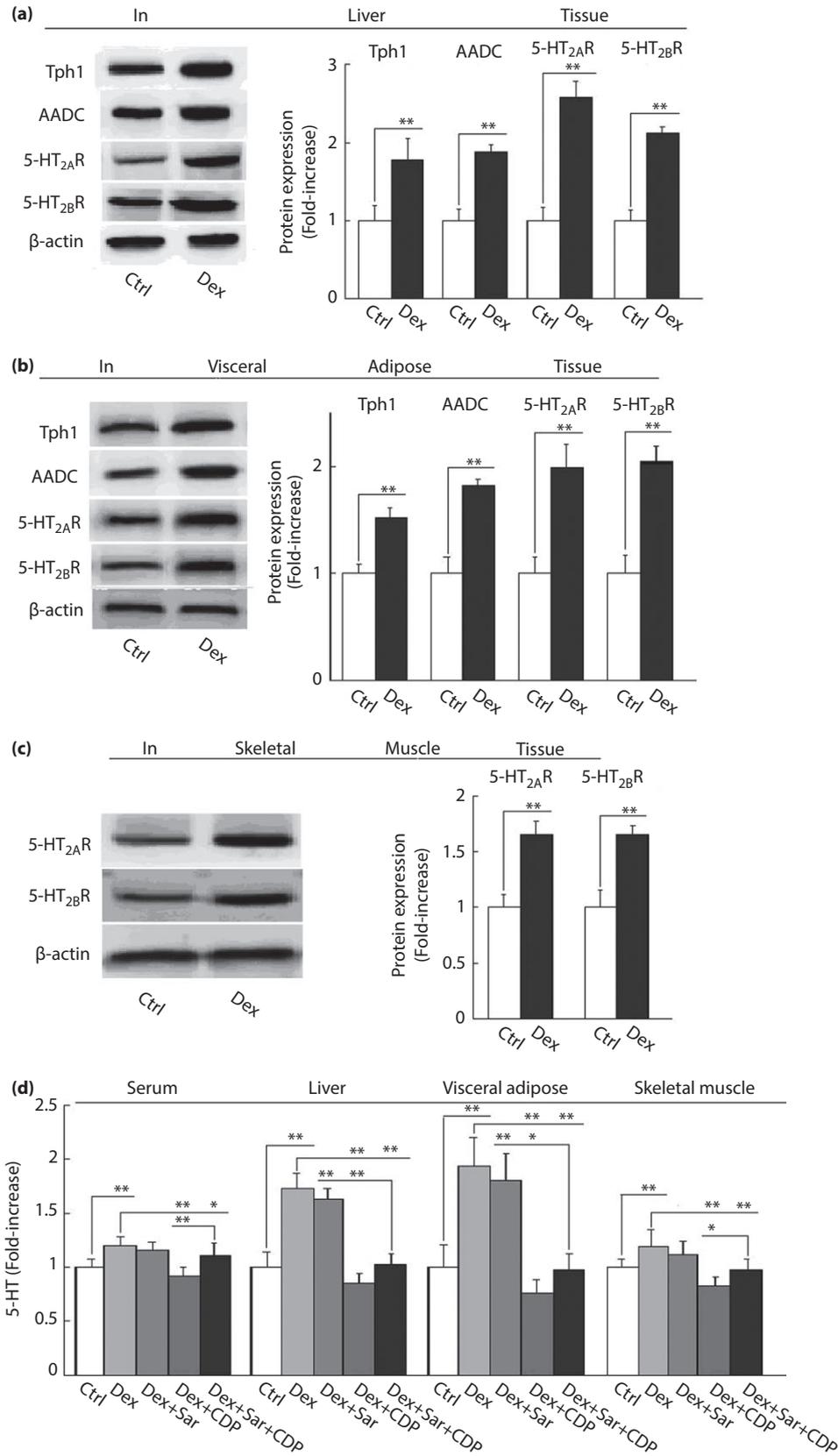
body IR, and to represent an important marker of cardiovascular risk, such as atherosclerosis, possibly even more so than visceral fat[38,39]. Though GC-induced lipolysis in white adipose tissue is controversial, GC promotes lipolysis by increasing messenger RNA expression of the two key lipolytic enzymes, hormone-sensitive lipase and ATGL, and has been found in adipose tissue through investigations of rats exposed to GC *in vivo* and adipocytes exposed to GC *in vitro*[33,40,41], and Cushing syndrome patients through microdialysis[42]. Increased lipolysis will result in elevated circulating FFA, which in turn can induce IR[40,43]. Though it is controversial as to whether glucose uptake is inhibited by GC in adipose tissue, one study showed that in the omental, but not in the subcutaneous, adipocytes in humans, GC decreases insulin-stimulated glucose uptake[44]. GC is also found to decrease insulin-mediated glucose uptake in the skeletal muscle, which can occur through stimulation of serine kinases, resulting in phosphorylation and inactivation of insulin receptor and insulin receptor substrate molecules[45]. In the present study, we found that GC-induced whole-body IR, and tissue-specific IR in the liver, visceral adipose and skeletal muscle are involved in enhanced 5-HT synthesis in the liver and visceral adipose, and upregulated $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2B}R$ in these tissues. Dex-induced IR can be abolished effectively by inhibition of both 5-HT_2R with Sar and peripheral 5-HT synthesis with CDP, and be strongly abolished by the combination of Sar and CDP in a synergistic manner, suggesting that the combination of Sar and CDP might be a dependable method for curing steroid-induced diabetes. Furthermore, the Sar or CDP effect was not owing to their impact on bodyweight or food intake, as Sar or/and CDP treatment resulted in a reversal to Dex-caused weight loss with a complete suppression to Dex-induced weight loss, but no reversal with a further decrease to the food intake in the rats. In addition to decreased food intake, the mechanisms of GC-caused weight loss are reduction in overall protein synthesis while promoting muscle proteolysis, and enhanced gluconeogenesis to result in hyperglycemia and glucose lost through urine[46,47]. Thus, we presume that Sar or/and CDP treatment also attenuates Dex-stimulated effects on protein metabolism, as well as suppressing gluconeogenesis. Additionally, though CDP is expected to lower dopamine production, Dex or CDP did not lead to a change in dopamine level in the present study, showing that dopamine is not the cause for GC-induced IR. We presume that a major action of AADC in the liver and visceral adipose is to assist 5-HT synthesis rather than dopamine.

It is presumed that the majority of 5-HT in the periphery is produced by the entero-chromaffin cells in the gut[48,49], and 5-HT is then majorly taken up and stored in platelets in circulation[50]. Dex-induced production of 5-HT in the entero-chromaffin cells has been observed in the small intestine, especially in the duodenum of rats[51]. However, both our previous study[22] and present study found that 5-HT is also synthesized in the liver and visceral adipose tissues, which were upregulated by Dex with upregulated expressions of both Tph1 and AADC,

and that increased 5-HT levels by Dex in both tissues came from tissue itself rather than the blood. 5-HT by acting on $5\text{-HT}_{2A}R$ mediates hepatic steatosis and IR has been shown in several studies[24,52], whereas activation of the mTOR–S6K pathway has been shown to be the mechanism of 5-HT action in the liver[53], and in the adipocytes and C2C12 myotubes by inhibition of insulin-stimulated activation of the IRS-1–AKT signaling pathway with glucose uptake[54]. The present study showed that Dex-induced activations of mTOR Ser^{2448} and S6K $\text{Thr}^{389/412}$ phosphorylation in the liver tissue were accompanied by upregulations of hepatic 5-HT synthesis, and $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2B}R$ expression, followed by hepatic IR, such as increased gluconeogenesis, downregulation of GLUT2 expression on the cell membrane, and increased TG and VLDL synthesis with steatosis in the liver tissue. More importantly, the aforementioned liver-IR markers induced by Dex could be abolished significantly by inhibition of $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2B}R$ with Sar or 5-HT synthesis with CDP, and were more effectively abolished by the combination of Sar and CDP, suggesting that GC-induced hepatic IR is closely involved in increased hepatic 5-HT synthesis and 5-HT_2R. In addition, the present study also suggested that Dex-stimulated lipolysis in the visceral adipose tissue resulted in increased serum FFA level, and downregulation of plasmalemmal GLUT4 expression in the visceral adipose and skeletal muscle tissue, and might very likely be involved in adipose-specific upregulation in 5-HT synthesis and 5-HT_2R expression, and muscle-specific upregulation in 5-HT_2R expression. We also found that it is both $5\text{-HT}_{2A}R$ and $5\text{-HT}_{2B}R$ instead of $5\text{-HT}_{2A}R$ alone[24,52] that mediate 5-HT-stimulated IR in the three tissues, both of which were upregulated by Dex. The precise mechanisms need to be studied further.

Taken together, peripheral 5-HT synthesis and 5-HT_2R ($5\text{-HT}_{2A}R$ and $5\text{-HT}_{2B}R$) are very important for GC-induced whole-body IR, and both of which can be upregulated by GC in the liver and visceral adipose with upregulated 5-HT_2R in the skeletal muscle. Inhibitions of both peripheral 5-HT synthesis and 5-HT_2R can be used in the treatment of GC-induced IR with diabetes. In addition, great elevation of HDL-c level in serum by the combination of Sar and CDP in the Dex-exposed rats, with great suppression on Dex-caused enhancement of LDL-c level in serum, showed that inhibition of both peripheral 5-HT synthesis and 5-HT_2R might also be an effective method for the treatment of atherosclerosis, as the goal of lowering total cholesterol and LDL-c, and raising HDL-c in blood has become a very important health issue for preventing or treating atherosclerosis[55].

DISCLOSURE

The authors declare no conflict of interest.

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