ORIGINAL ARTICLE

Important role of 5-hydroxytryptamine in glucocorticoid-induced insulin resistance in liver and intra-abdominal adipose tissue of rats

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ABSTRACT

Aim/Introduction: Both glucocorticoids and 5-hydroxytryptamine (5-HT) have been shown to induce insulin resistance (IR) in hepatocytes and adipocytes. Here, we explore whether there is a correlation between them.

Materials and Methods: Except for the control group, male rats were exposed to dexamethasone treated with or without para-chlorophenylalanine (pCPA), or carbidopa for 20 days. Except for the control group, buffalo rat liver 3A (BRL-3A) cells were exposed to dexamethasone for 24 h, treated with or without pCPA, carbidopa, or clorgiline for 48 h, or exposed to 5-HT treated with or without fluoxetine for 48 h. Whole-body IR was determined by both glucose tolerance test and measurement of fasting blood glucose and insulin, whereas hepatocytes or adipocytes IR was determined by examining either hepatic gluconeogenesis, steatosis and glucose transporter 2 expression or lipolysis.

Results: Dexamethasone-induced whole-body IR, liver and intraabdominal adipose IR were accompanied by upregulated expressions of tryptophan hydroxylase-1 and aromatic amino acid decarboxylase with increased 5-HT level in both tissues, which were attenuated significantly by pCPA, inhibiting tryptophan hydroxylase-1, or carbidopa, inhibiting aromatic amino acid decarboxylase. [Correction added on 22 September 2015, after first online publication: 'inhibiting aromatic amino acid decarboxylase' was duplicated and has been replaced by 'tryptophan hydroxylase-1'.] In the BRL-3A cells, dexamethasone-induced IR was also accompanied by upregulated 5-HT synthesis in dose- and time-dependent manners, and was attenuated by pCPA or carbidopa, but exacerbated by clorgiline, inhibiting monoamine oxidase-A to further increase 5-HT level. Dexamethasone also enhanced 5-HT 2A and 2B receptor expressions in both tissues and BRL-3A cells. Additionally, blocking 5-HT transporter with fluoxetine significantly suppressed 5-HT-induced IR in BRL-3A cells.

Conclusion: Enhancement of 5-HT synthesis in liver and intra-abdominal adipose is an important reason for glucocorticoids-induced IR.

INTRODUCTION

Glucocorticoids (GCs) are potent agents used to treat a range of human diseases, including autoimmune diseases and cancer, and to prevent rejection after organ transplantation. Although lifesaving for many, they come at a high price, often leading to obesity, insulin resistance (IR), diabetes and osteoporosis[1]. There is sufficient evidence in several species showing that GCs result in an increase in serum insulin and glucose[2–6], and a decrease in insulin-mediated glucose uptake[1,7]. In addition, chronic treatment with glucocorticoid drugs, such as dexamethasone (Dex), has been associated with hyperinsulinemia in both animal and human studies[8,9]. GC-induced whole-body IR is tightly correlated to its metabolic effects in individual organs[10]. GC-induced metabolic effects have focused on the liver, skeletal muscle and adipose tissue. It has

been shown that the metabolic events occurring in these tissues play a key role in the occurrence of whole-body abnormalities in metabolic syndrome.

A recent study showed that 5-hydroxytryptamine (5-HT) is an essential component in the exacerbation of hepatic steatosis in L-tryptophan-treated mice fed with a high-fat and high-fructose diet[11]. 5-HT can also induce IR in cultured 3T3-L1 adipocytes, mouse primary adipocytes and C2C12 myotubes[12]. It is widely believed that the majority of 5-HT in the periphery is synthesized by enterochromaffin cells, a type of epithelial cell in the gastrointestinal tract, and exported to peripheral organs through circulation[13]. L-Tyrosine is the main substrate for serotonin biosynthesis in mammals, which is oxidized to 5-hydroxy-L-tryptophan by tryptophan hydroxylase (Tph), first, then further decarboxylated to 5-HT by aromatic amino acid decarboxylase (AADC)[14]. Two subtypes of Tph are identified, namely Tph2 found in the central nervous system and Tph1 found in the peripheral systems (Tph1).

Whether GC-induced IR in the liver and intra-abdominal adipose has an association with 5-HT remains unclear. To explore this issue, we carried out the present study using a Dex-induced IR model in the rats and rat hepatocyte lines. We found that both liver and intra-abdominal adipose in the rats synthesize 5-HT, which is upregulated by chronic Dex exposure, and is an important reason for Dex-induced IR in whole-body and both tissues.

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. Male Sprague–Dawley rats (10 weeks old and weighing 250–300 g, supplied by B&K Universal Group Limited, Shanghai, China) were randomly divided into four groups ($n = 6$ per group): the control group, Dex (Cisen Pharmaceutical Ltd, Jining, China) exposure, Dex exposure with para-chlorophenylalanine (pCPA; an inhibitor of Tph1; TCI, Tokyo, Japan) treatment, or Dex exposure with carbidopa (CDP; an inhibitor of AADC; Sigma, St. Louis, MO, USA) treatment for 20 days. Except for control rats, the others were subcutaneously administered with Dex (0.75 mg/kg twice daily on the morning and afternoon with an interval of 12 h), whereas the control rats were subcutaneously administered with an equivalent volume of normal saline. At 60 min before Dex treatment, pCPA-treated rats were subcutaneously administered with pCPA (7.5 mg/kg twice daily) according to Jensen et al.[15], and CDP-treated rats were intragastrically administered with CDP (10 mg/kg twice daily), whereas rats in the other groups were subcutaneously or intragastrically administered with an equivalent volume of normal saline or 0.5% CMC-Na, respectively. Bodyweight and food-intake were recorded for 5 days. On the 18th day of treatment, the intraperitoneal glucose tolerance test (IPGTT) was carried out at 12 h after fasting, and 5 h after the drug and Dex administration. 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. Blood samples were collected and centrifuged (600 g, for 10 min) to obtain serum. Livers, intra-abdominal adipose (mesenteric, bilateral perirenal and epididymal) and thigh muscle tissue were removed immediately, washed in cold phosphate-buffered saline (PBS) and weighed. Hepatic index (HI; HI = liver weight / bodyweight × 100%) and visceral fat index (VFI; VFI = intra-abdominal adipose weight / bodyweight × 100%) were calculated. Samples of serum and the tissues were stored at −80°C immediately for further experiment progress.

Cell culture and treatments
Rat normal liver cell line buffalo rat liver 3A cells (BRL-3A cells) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured with PRMI-1640 (Gibco, Grand Island, NY, USA) medium 10% (v/v) fetal bovine serum (Biological Industries, Gaza, Israel) and penicillin/streptomycin (100 U/mL, respectively) at a condition of 37°C, 5% CO_2. After cells were confluent to 70–80% in the flask bottom, medium was removed and fresh serum-free medium containing drugs was added and incubated for another 24–48 h, then harvested for further experimental processes. All experiments were repeated at least three times.

The cells were divided into 10 groups: (i) control for 24 h (group 1); (ii) 30 μmol/L Dex exposure for 24 h (group 2); (iii) control for 48 h (group 3); (iv) 30 μmol/L pCPA exposure for 48 h (group 4); (v) 30 μmol/L CDP exposure for 48 h (group 5); (vi) 10 μmol/L Dex exposure for 48 h (group 6); (vii) 30 μmol/L Dex exposure for 48 h (group 7); (viii) 30 μmol/L Dex and 30 μmol/L pCPA exposure for 48 h (group 8); (ix) 30 μmol/L Dex and 30 μmol/L CDP exposure for 48 h (group 9); (x) 30 μmol/L Dex and 5 μmol/L clorgiline (CGN; an inhibitor of monoamine oxidase-A [MAO-A]; Sigma) exposure for 48 h (group 10). The pCPA, CDP or CGN-treated cells were pretreated with them, respectively, 30 min before Dex exposure. In another trial, the cells were divided into three groups: (i) incubated with normal medium for 48 h; (ii) incubated with 30 μmol/L 5-HT (Alfa-Aesar, Ward Hill, MA, USA)-containing medium for 48 h; or (iii) incubated with 30 μmol/L 5-HT and 30 μmol/L fluoxetine (Flx; an inhibitor of 5-HTT; Sigma)-containing medium for 48 h. The Flx-treated cells were pretreated with Flx 30 min before 5-HT exposure.

IPGTT
A dose of 2 g/kg glucose was injected. Blood was sampled at 0, 30, 60 and 150 min by tail bleeding. Blood glucose was measured in using a LifeScan Blood Glucose Meter (Johnson & Johnson, New Brunswick, NJ, USA). The IPGTT was evaluated by the total area under blood glucose curve using the trapezoidal method[16].

Serum and hepatic biochemical analysis

Sample preparation involved liver or adipose tissues being sliced to pieces weighing 0.4 g, then homogenized in 4 mL cold PBS buffer, and the homogenate was used for detection; for BRL-3A cells, cells were washed and scraped with PBS, and incubated with radio immunoprecipitation assay buffer for cell lysis, then used for detection.

The level of 5-HT and insulin was measured by using an enzyme-linked immunosorbent assay kit (Abcam, Hong Kong); the levels of triglycerides (TGs), free fatty acids, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and glucose in the serum, and TGs, H_2O_2 and glycerinum in the tissue and BRL-3A cells were measured by using a spectrophotometer kit (Nanjing Jianchen, Nanjing, China).

Oil Red O staining in the liver tissue

The liver was placed in an optimal cutting temperature chamber overnight at $-80°C$, and 6-μm sections were made. Tissue slices were rinsed with PBS, raised with 60% isopropanol and stained with freshly prepared Oil Red O working solution for 15 min. Tissue slices were rinsed with 60% isopropanol again and counterstained with hematoxylin for nuclei. For cells, the BRL-3A cells were fixed with 10% formalin and then stained with Oil Red O.

Reverse transcription polymerase chain reaction

Total ribonucleic acid (RNA) was extracted from BRL-3A cells, liver tissue, intra-abdominal adipose tissue or thigh muscle tissue using RNAiso Plus Isolation Reagent (TAKARA, Otsu, Shiga, Japan) following the manufacturer's instructions. Total RNA was reserve transcribed and amplified in a GeneAmp polymerase chain reaction (PCR) system (Eppendorf, Hamburg, Germany); reverse transcription PCR for Tph1, AADC, glycerin-3-phosphate acyltransferase 1 (GPAT1), phosphoenolpyruvate carboxykinase-1 (PEPCK-1), and glucose transporter 2 (GLUT2) in the liver and BRL-3A cells, 5-HT 2A and 2B receptor (5-HT$_{2A}$R and 5-HT$_{2B}$R) in the BRL-3A cells, Tph1, AADC and adipose triglyceride lipase in the intra-abdominal adipose, and Tph1 and AADC in the thigh muscle were carried out in the same system. 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 all genes were adjusted to glyceraldehyde 3-phosphate dehydrogenase. The relative expression levels of each gene were calculated as: (relative gray value of each gene / mean of relative gray value in the control) × 100%. PCR primer sequences are listed in Table 1.

Western blotting

BRL-3A cells, liver tissue or intra-abdominal adipose tissue were homogenized in lysis buffer, then sonicated and incubated on ice for 15 min. Protein was extracted by Cytosol and Membrane Protein Extraction Kit (Beyotime Institute of Biotechnol-

Table 1 | Primers used for reverse transcription polymerase chain reaction

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GAPDH	TATCGGACGCCTGGTTAC	TGCTGACAATCTTGAGGGA
Tph1	ACTGCGACATCAACCGAGAA	GGCTAACCCTGACAGGAAAT
AADC	AGAACTGACCTAACCGAAGC	CAGACCAACCCGAGGATGAC
5-HT$_{2A}$R	GGATTTACCTGGATGTGC	TGGATGGACCGTTGGAAG
5-HT$_{2B}$R	CAGCAGCAGAGGGAAATGA	ATCCAGGGAAATGGCACA
PEPCK-1	ACAGCTCATGTTTTGTTTGG	GGTTGAAAAACC TTT CCC AG
ATGL	TTCAAGTTTCCTTGCAGAGT	CTCCCAAACTGACCCTTAAA
GLUT2	GGTGTTGCTGGATAAGTTCA	AGGATTCGAGTTAAGAGGGA
GPAT1	ACAGCTCATGTTTTGTTTGG	GGTTGAAAAACCTTTCCCAG

5-HT$_{2A}$R, 5-hydroxytryptamine 2A receptor and 2B receptor; 5-HT$_{2B}$R, 5-hydroxytryptamine 2B receptor; AADC, aromatic amino acid decarboxylase; ATGL, adipose triglyceride lipase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT2, glucose transporter 2; GPAT1, glycerin-3-phosphate acyltransferase 1; PEPCK-1, phosphoenolpyruvate carboxykinase-1; Tph1, tryptophan hydroxylase 1.

ogy, 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: Tph1 and AADC (Epitomics-Abcam, Hong Kong), PEPCK-1 (Signalway Antibody LLC, College Park, MD USA), GPAT1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), GLUT2 (Epitomics-Abcam), 5-HTR$_{2A,2B}$Rs (GeneTex Inc, Irvine, CA, USA) and β-actin (Bioworld Technology Inc, Shangahi, China). Then the membranes were incubated with the horseradish peroxidase-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 expression levels of each protein were calculated as: (relative gray value of each protein / mean of relative gray value in the control) × 100%.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Data differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA) followed by least significant difference multiple comparison test, while the differences of messenger RNA (mRNA), and protein levels of Tph1 and AADC in the liver, intra-abdominal adipose, and BRL-3A cells were tested by using Student's t-test. $P < 0.05$ was considered significant.

RESULTS

Dex-induced whole-body insulin resistance correlates with Dex-stimulated peripheral 5-HT synthesis

By the end of the 20-day experiment, bodyweight and food intake in the Dex-exposed rats decreased markedly (Figure 1a,
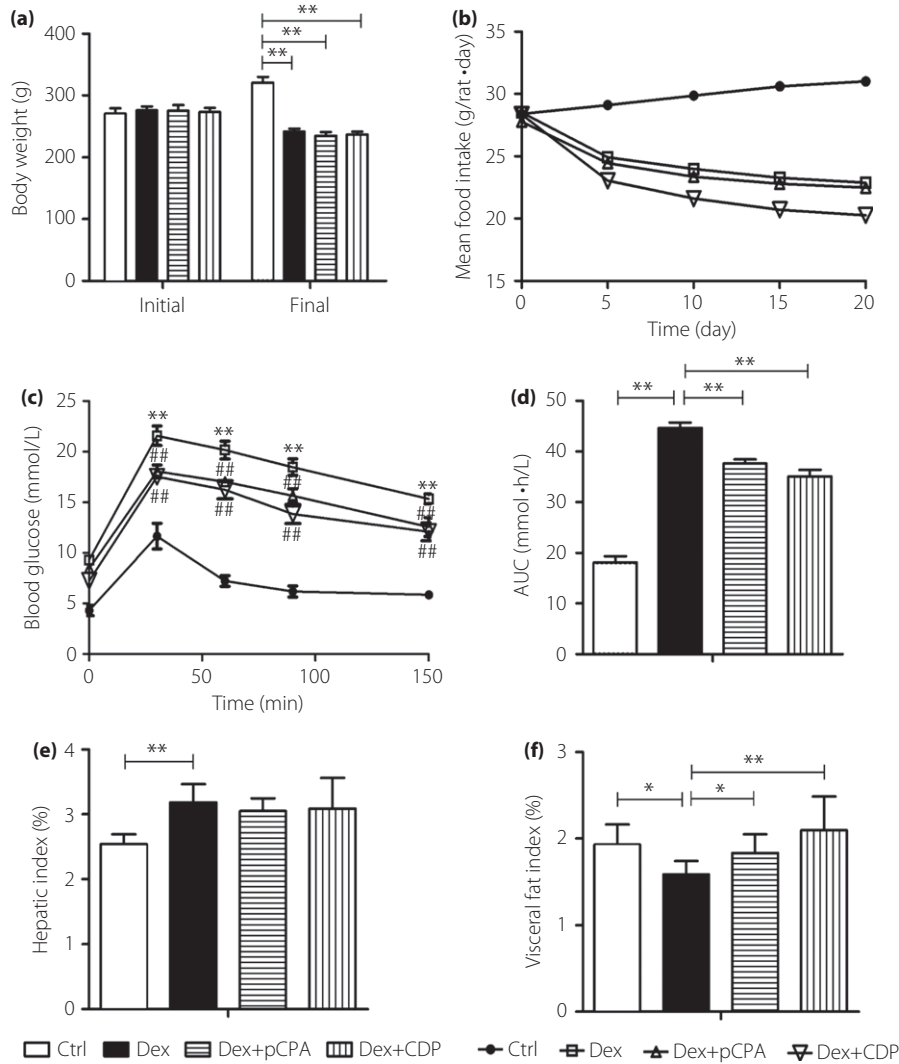
Figure 1 | (a) Bodyweight, (b) food intake, (c) blood glucose levels and (d) total area under blood glucose curve (AUC) after intraperitoneal injection of glucose (2.0 g/kg), (e) hepatic index and (f) visceral fat index in the groups of control (Ctrl), dexamethasone exposure (Dex), Dex exposure with 4-chloro-DL-phenylalanine (pCPA) treatment (Dex+pCPA) and Dex exposure with carbidopa (CDP) treatment (Dex + CDP). The results were shown as mean ± standard deviation (n = 6 per group). (c) **$P < 0.01$ vs the control; ##$P < 0.01$ vs the Dex. (a,b,d–f) *$P < 0.05$; **$P < 0.01$.

b). pCPA or CDP treatment did not reverse bodyweight decrease, but CDP treatment further deceased food intake in the Dex-exposed rats (Figure 1a,b). To examine Dex-induced whole-body IR, IPGTT, fasting blood glucose and insulin were measured. We found that rats chronically exposed to Dex resulted in marked glucose intolerance, hyperglycemia and hyperinsulinemia (Figures 1c,d and 2c,d) after an increase of serum 5-HT level (Figure 3c). Dex-induced effects were attenuated significantly by either pCPA or CDP treatment (Figures 1c,d, 2c,d and 3c), suggesting that Dex-induced whole-body IR is associated with enhancement of peripheral 5-HT synthesis. We also detected a Dex-caused HI increase (Figure 1e), a marker of hepatic steatosis, and VFI decrease (Figure 1f), a marker of visceral fat mass, and dyslipidemia, including increased serum level of TGs, low-density lipoprotein

cholesterol and free fatty acids, and decreased serum level of high-density lipoprotein cholesterol (Figure 2a,b). Either pCPA or CDP treatment showed a decreased tendency to Dex-induced HI increase (Figure 1e), and reversed Dex-induced VFI decrease (Figure 1f) and dyslipidemia (Figure 2a,b), suggesting that Dex-induced alteration of fat distribution in the liver and intra-abdominal adipose depot, and dyslipidemia is also associated with enhancement of peripheral 5-HT synthesis.

5-HT synthesis is found in the rat liver and Intra-abdominal adipose, and is upregulated by chronic Dex exposure

To examine whether there is 5-HT synthesis in the liver, intra-abdominal adipose and skeletal muscle of rats, gene or protein expressions of Tph1 and AADC with 5-HT content were measured. We detected expression of Tph1 and AADC in the liver
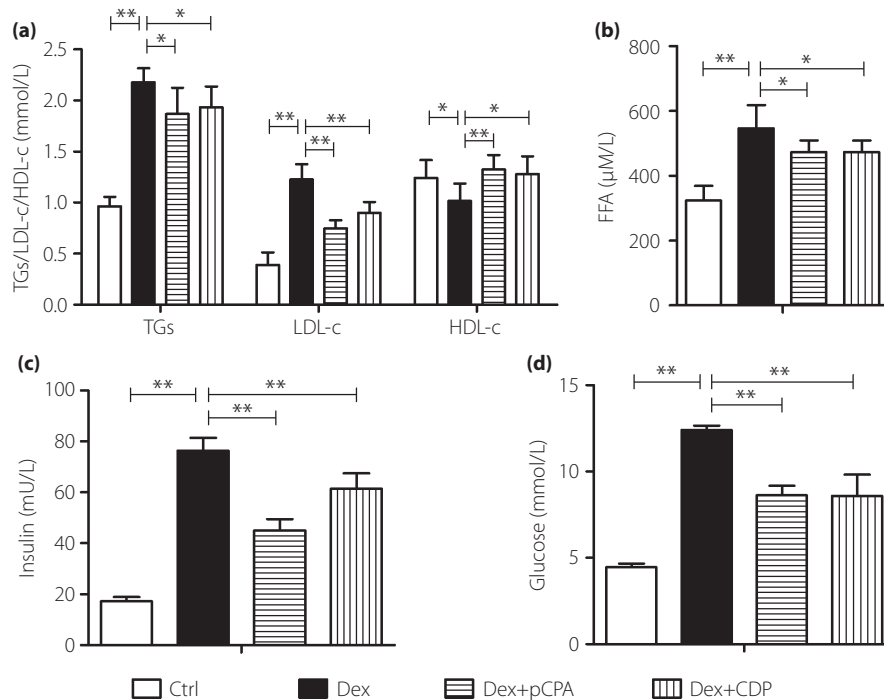
Figure 2 | (a) Triglycerides (TGs), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) levels, (b) free fatty acids (FFA) levels, (c) insulin levels and (d) glucose levels in the serum. In the experiment, rats were treated with normal (Ctrl) or dexamethasone exposure (Dex), Dex exposure with 4-chloro-DL-phenylalanine (pCPA) treatment (Dex+pCPA), or Dex exposure with carbidopa (CDP) treatment (Dex + CDP) for 20 days. Data shown as mean ± standard deviation (n = 6). *P < 0.05; **P < 0.01.

and intra-abdominal adipose, whereas they were not detected in the skeletal muscle (Figure 3a). Chronic Dex exposure increased expression of Tph1 and AADC in both the liver and intra-abdominal adipose tissues (Figure 3a,b). Meanwhile, 5-HT levels in both tissues and serum were also elevated by Dex (Figure 3c). Treatment with either pCPA, inhibiting Tph1, or CDP, inhibiting AADC, fully abolished Dex-induced enhancement of 5-HT level in both tissues and serum (Figure 3c). However, Dex-induced 5-HT increases in both tissues and serum were very different; for example, the fold increase of 5-HT vs control was 0.65 in the liver and 0.91 in the intra-abdominal adipose, whereas the fold increase was 0.19 in the serum (Figure 3c), suggesting that the high 5-HT level in the liver and intra-abdominal adipose induced by Dex originated from upregulated 5-HT synthesis in both tissues rather than from the serum. These results showed that there is 5-HT synthesis in both the liver and intraabdominal adipose, which is increased by long-term Dex exposure.

Upregulated 5-HT synthesis with elevation of 5-HT level is distinctly involved in Dex-induced IR in the liver and intra-abdominal adipose

GC-induced IR in the liver can be determined by examination of glucose uptake, gluconeogenesis and steatosis, whereas that in adipose tissue can be determined by examining lipolysis[13]. In the present study, rats chronically exposed to Dex obviously

resulted in IR in the liver with upregulated mRNA and protein expressions of PEPCK-1 and GPAT1 (Figure 4a,b), increased hepatic TGs content and steatosis (Figure 4c,f), and downregulated mRNA and protein expressions of GLUT2 (Figure 4a,b). Furthermore, Dex-induced IR in the intra-abdominal adipose was also observed with upregulated mRNA expression of adipose triglyceride lipase, and elevated glycerinum level (Figure 4d,e). Either pCPA or CDP treatment markedly suppressed Dex-induced effects (Figure 4a–f). The results showed that Dex-induced IR in the liver and intra-abdominal adipose is associated with Dex-caused elevation of 5-HT levels in both tissues.

Dex-induced IR in BRL-3A cells was also involved in upregulation of 5-HT synthesis with elevation of 5-HT Level

The mRNA and protein expressions of Tph1 and AADC with 5-HT content were detected in the cultured BRL-3A cells in vitro (Figure 5a–c), suggesting 5-HT synthesis in the BRL-3A cells, while pCPA or CDP treatment significantly decreased the 5-HT level in the cells (Figure 5c), Dex exposure significantly upregulated expression of Tph1 and AADC with elevated 5-HT level in dose- and time-dependent manners in the cells (Figure 5a–c). The Dex-stimulated increase of 5-HT level in the BRL-3A cells was completely suppressed, with even lower 5-HT levels than that in the control, by either pCPA or CDP treatment, while further elevated by CGN (Figure 5c), a
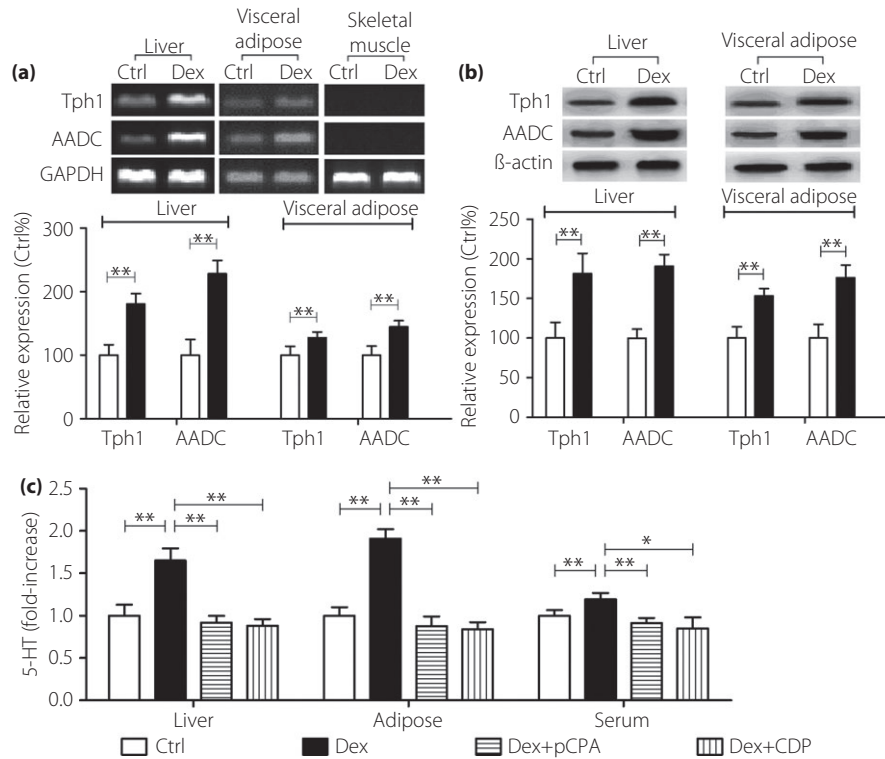
Figure 3 | (a) Messenger ribonucleic acid expressions of tryptophan hydroxylase 1 (Tph1) and aromatic amino acid decarboxylase (AADC) in the liver, intra-abdominal adipose- and thigh muscle, (b) protein expressions of Tph1 and AADC in the liver and intra-abdominal adipose, and (c) 5-hydroxytryptamine (5-HT) levels in the liver, intra-abdominal adipose and serum. In the experiment, rats were treated with normal (Ctrl) or dexamethasone exposure (Dex), Dex exposure with 4-chloro-DL-phenylalanine (pCPA) treatment (Dex + pCPA), or Dex exposure with carbidopa (CDP) treatment (Dex + CDP) for 20 days. Data shown as mean ± standard deviation ($n = 6$). *$P < 0.05$; **$P < 0.01$.

MAO-A inhibitor that reduces the ability of MAO-A for degrading 5-HT. Meanwhile, Dex induced IR in the BRL-3A cells in time- and dose-dependent manners, with time- and dose-dependent enhancement of PEPCK-1 and GPAT1 mRNA expression, TGs level, and time- and dose-dependent reduction of GLUT2 mRNA expression (Figure 5d,e). Dex-stimulated effects were attenuated obviously by either pCPA or CDP treatment, but were exacerbated by CGN treatment (Figure 5d,e). In addition, marked lipid droplets examined by Oil Red O staining in all treatment groups were not found (data not shown). The data showed that Dex-induced IR is closely associated with increased 5-HT level in the BRL-3A cells.

5-HT-induced IR is also associated with 5-HT uptake in the BRL-3A cells

In the present study, cultured BRL-3A cells were treated with 5-HT with or without Flx treatment, a 5-HTT inhibitor that restrains 5-HT uptake through 5-HTT in the cell. 5-HT exposure showed similar effects with Dex exposure, with upregulated expression of GPAT1 and PEPCK-1 mRNA, elevated TGs level, and downregulated expression of GLUT2 mRNA, which were attenuated significantly by Flx treatment. Nevertheless, Flx failed to fully abolish 5-HT effects in the cells, suggesting that, besides 5-HT entering into the cells, there exists others that

mediate IR. In addition, marked lipid droplets examined by Oil Red O Staining were not found in all groups (data not shown). The results suggested that, similar to Dex exposure, exogenous 5-HT action also involves 5-HT-induced IR in the BRL-3A cells by entering into the cells through 5-HTT. The results are shown in Figure 6.

DISCUSSION

The present study examined the contribution of 5-HT to Dex-induced IR, focusing mainly on the liver and intra-abdominal adipose. GC exposure in humans is well known to result in whole-body IR, and GC-induced IR has also been confirmed when animals were chronically exposed to excess GC[17]. In the present study, long-term Dex overexposure resulted in whole-body IR in rats, with significant glucose intolerance, hyperglycemia, hyperinsulinemia and dyslipidemia. In addition, Dex-caused metabolic disorders, which are markers of IR, were also detected in the liver and intra-abdominal adipose. In the liver, Dex resulted in consequences with upregulating PEPCK-1 expression, which is an indication of increased gluconeogenesis[18]; upregulating GPAT1 expression with high TGs content and steatosis, which is an indication of increased TGs synthesis[19]; and downregulating GLUT2 expression, which is an indication of decreased glucose uptake[20]. In the intra-abdominal
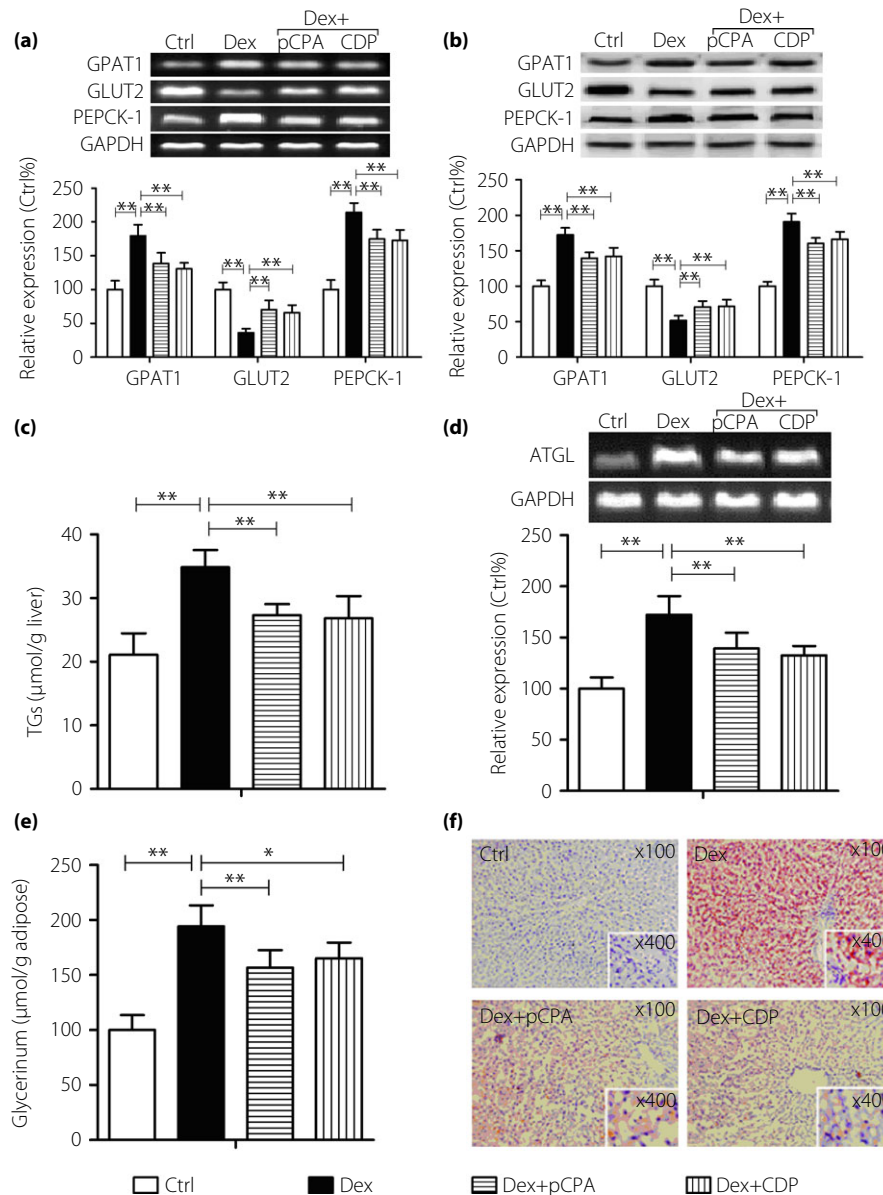
Figure 4 | (a) Messenger ribonucleic acid expressions of glycerin-3-phosphate acyltransferase 1 (GPAT1), glucose transporter 2 (GLUT2) and phosphoenolpyruvate carboxykinase-1 (PEPCK-1) in the liver, (b) protein expressions of GPAT1, GLUT2 and PEPCK-1 in the liver, (c) triglycerides (TGs) levels in the liver, (d) messenger ribonucleic acid expression of adipose triglyceride lipase (ATGL) in the intra-abdominal adipose, (e) glycerinum levels in the intra-abdominal adipose and (f) representative Oil Red O staining pathological images in the liver. In the experiment, rats were treated with normal (Ctrl) or dexamethasone exposure (Dex), Dex exposure with 4-chloro-DL-phenylalanine (pCPA) treatment (Dex + pCPA), or Dex exposure with carbidopa (CDP) treatment (Dex + CDP) for 20 days. Data shown as mean ± standard deviation ($n = 6$). *$P < 0.05$; **$P < 0.01$.

adipose, Dex resulted in upregulation of adipose TG lipase expression with high glycerinum levels in the adipose and high free fatty acids level in serum, which is an indication of increased lipolysis[21]. These disorders are typical markers of GC-induced IR[17]. More importantly, we found that 5-HT was synthesized in both organs with the expression of 5-HT-synthetic enzymes, Tph1 and AADC, which were also detected in the cultured BRL-3A cells *in vitro*. Dex-induced whole-body,

liver and intra-abdominal adipose IR were in line with Dex-stimulated 5-HT synthesis in both tissues, and was attenuated significantly by decreasing 5-HT levels with inhibition of Tph1 or AADC. Similar results were detected in the BRL-3A cells exposed to Dex with or without inhibition of Tph1 or AADC. Furthermore, as contrary evidence for inhibiting 5-HT synthesis, inhibition of MAO-A activity, resulting in a higher 5-HT level in the BRL-3A cells, exacerbated Dex-induced IR. These
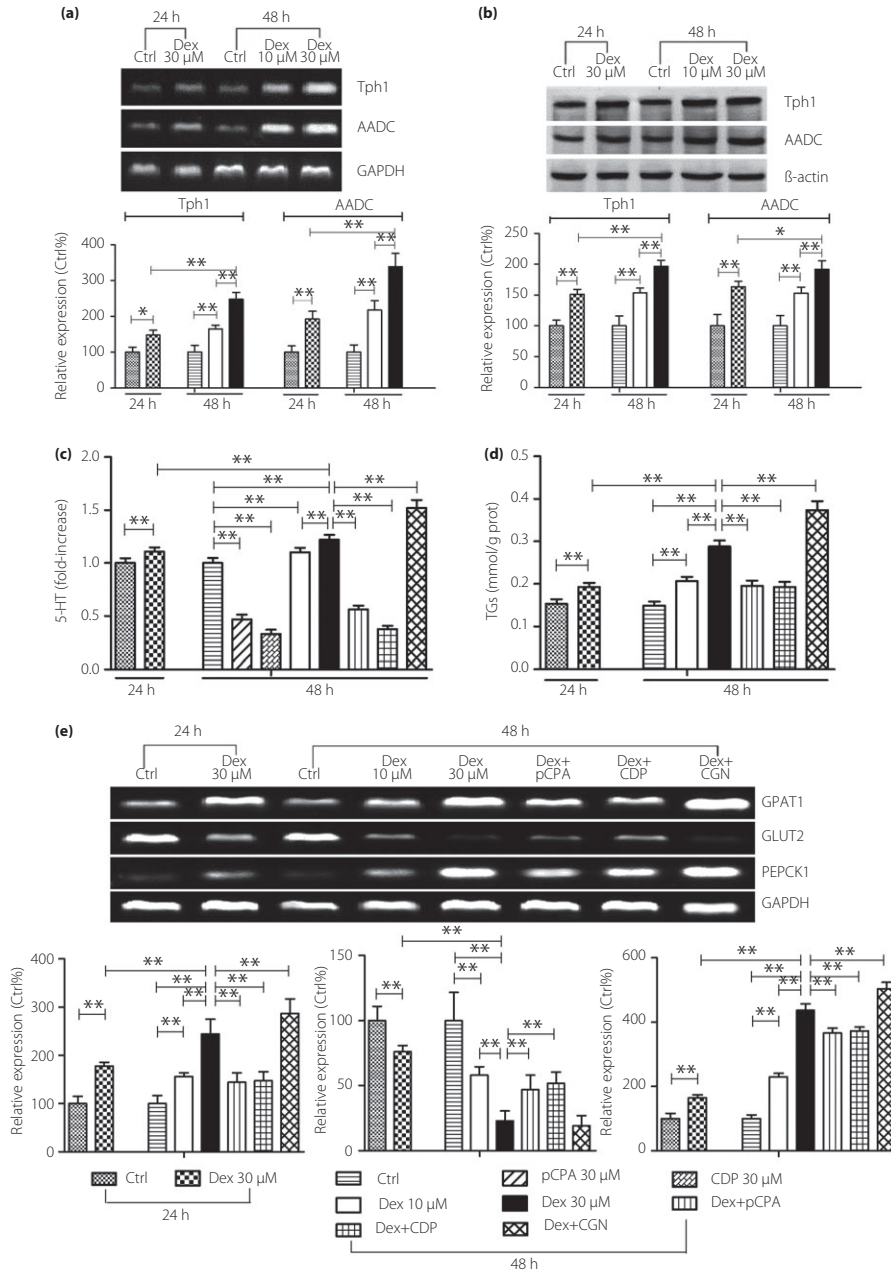
Figure 5 | (a) Messenger ribonucleic acid and (b) protein expressions of tryptophan hydroxylase 1 (Tph1) and aromatic amino acid decarboxylase (AADC), (c) 5-hydroxytryptamine (5-HT) levels, (d) triglycerides (TGs) levels and (e) messenger ribonucleic acid expressions of glycerin-3-phosphate acyltransferase 1 (GPAT1), glucose transporter 2 (GLUT2) and phosphoenolpyruvate carboxykinase-1 (PEPCK-1) in the buffalo rat liver 3A cells. In the trials, buffalo rat liver 3A cells were incubated with normal medium (Ctrl) or 30 μmol/L dexamethasone-containing medium (Dex 30 μmol/L) for 24 h, normal medium (Ctrl), 30 μmol/L 4-chloro-DL-phenylalanine-containing medium (pCPA 30 μmol/L), 30 μmol/L carbidopa-containing medium (CDP 30 μmol/L), 10 μmol/L Dex-containing medium (Dex 10 μmol/L), 30 μmol/L Dex-containing medium (Dex 30 μmol/L), 30 μmol/L Dex and 30 μmol/L pCPA-containing medium (Dex+pCPA), 30 μmol/L Dex and 30 μmol/L CDP-containing medium (Dex + CDP), or 30 μmol/L Dex and 5 μmol/L clorgiline (CGN)-containing medium (Dex + CGN) for 48 h. Data shown as mean ± standard deviation ($n = 3$ per group). *$P < 0.05$; **$P < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

results strongly suggest a novel finding that activated 5-HT synthesis with an increase in 5-HT level induced by GC in the liver and intra-abdominal adipose is significantly involved in

GC-induced IR. In addition, the contribution of 5-HT to the GC-induced decrease of bodyweight and appetite is slight, because pCPA or CDP treatment, reducing 5-HT levels in the
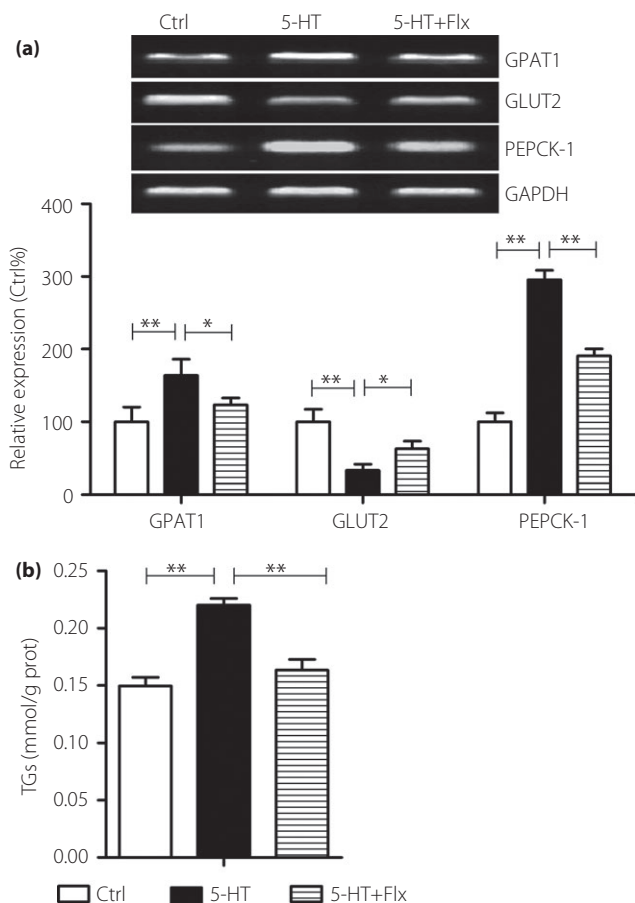
Figure 6 | (a) Alterations of messenger ribonucleic acid expressions of glycerin-3-phosphate acyltransferase 1 (GPAT1), glucose transporter 2 (GLUT2) and phosphoenolpyruvate carboxykinase-1 (PEPCK-1), and (b) triglycerides (TGs) contents in the buffalo rat liver 3A cells exposed with 5-hydroxytryptamine (5-HT) with or without fluoxetine (Flx) treatment. In the trials, buffalo rat liver 3A cells were incubated with normal medium (Ctrl), 30 μmol/L 5-HT-containing medium (5-HT), 30 μmol/L 5-HT and 30 μmol/L Flx-containing medium (5-HT + Flx) for 48 h. Data shown as mean ± standard deviation ($n = 3$). *$P < 0.05$; **$P < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

serum, liver and intra-abdominal adipose, did not change the Dex-induced bodyweight decrease, and only CDP could further decrease the Dex-caused food intake decrease.

To date, the mechanisms of GC-induced IR in the liver and adipose tissue still remain to be elucidated. The present study showed that GC-induced IR in the liver and intra-abdominal adipose is, at least in part, caused by GC-induced 5-HT increase by upregulating 5-HT synthesis. The mechanisms by which 5-HT results in IR in the hepatocytes and adipocytes have been shown. 5-HT through activating mammalian target of rapamycin signaling stimulates lipogenesis in the liver, and leads to serine phosphorylation of insulin receptor substrate-1 to impair insulin signal transduction in the adipocytes[11,12]. '5-HT by acting on its multiple receptors

exerts its effects to cells' is widely accepted. 5-HT 2 receptors in the hepatocyte and adipocyte are recognized as major receptors in 5-HT-induced IR[12,22,23]. Indeed, we also detected upregulated expression of $5\text{-}HT_{2A}R$ and $5\text{-}HT_{2B}R$ induced by Dex in the live, intra-abdominal adipose and BRL-3A cells (data in Figures S1 and S2). However, the present results showed that 5-HT has an intracellular action when it induces IR, because 5-HT-induced IR with upregulation of PCPCK-1 and GPAT1 expression, elevation of TGs level, and downregulation of GLUT2 expression in the BRL-3A cells were attenuated significantly by Flx, which restrains 5-HT uptake through 5-HTT, though without complete suppression. In addition, though it is reported that increased reactive oxygen species by 5-HT, which owes to 5-HT broken by MAO-A, is a major reason for 5-HT-induced steatohepatitis in the choline methionine-deficient diet-fed mice[24]. This 5-HT effect seems not to be a key reason for 5-HT-induced IR in the hepatocytes, as, in the present trial, Dex-caused IR in the BRL-3A cells was exacerbated by inhibiting MAO-A, which increased 5-HT levels, but decreased H_2O_2 levels (data in Figure S3), a major reactive oxygen species produced by MAO-A when it degrades 5-HT.

Taken together, the present study reveals the fact that 5-HT is synthesized both in the liver and intra-abdominal adipose, which is activated by chronic GC overexposure, and is an important reason for GC-induced IR in both organs and whole body. GC also upregulates 5-HT 2 receptors in both organs, which might be another reason for GC-induced IR. Whether there is a synergistic effect on GC-induced IR between increased 5-HT synthesis and enhanced 5-HT 2 receptors requires further investigation. The present study provides evidence that 5-HT might represent a novel target for the prevention and treatment of GC-induced IR.

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | Messenger ribonucleic acid and protein expression of 5-hydroxytryptamine(5-HT) 2A (5-HT$_{2A}$R) and 2B receptor (5-HT$_{2B}$R) in rats' liver. Ctrl, control; Dex, dexamethasone.

Figure S2 | Messenger ribonucleic acid and protein expression of 5-hydroxytryptamine (5-HT) 2A and 2B receptor in buffalo rat liver 3A (BRL-3A) cells. Ctrl, control; Dex, dexamethasone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure S3 | H$_2$O$_2$ levels in buffalo rat liver 3A cells. Ctrl, control; Dex, dexamethasone.