3-Deoxyglucosone induces insulin resistance by impairing insulin signaling in HepG2 cells

GUOQIANG LIANG*, FEI WANG*, XIUDAO SONG, LURONG ZHANG, ZHEN QIAN and GUORONG JIANG

Basic Research Laboratory, Suzhou Academy of Wumen Chinese Medicine, Suzhou Hospital of Traditional Chinese Medicine, Suzhou, Jiangsu 215003, P.R. China

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Abstract. 3-Deoxyglucosone (3DG), a highly reactive dicarbonyl intermediate generated during glycation, has been confirmed to be markedly elevated in the plasma of patients with diabetes. Our previous study found that there is an association between increasing accumulation of plasma 3DG and impaired glucose regulation in non-diabetic seniors (females, >50 years old; males, >55 years old). It was also found that 3DG led to impaired plasma glucose homeostasis in healthy mice, however, the mechanisms underlying the deleterious effect of 3DG in diabetes remain to be fully elucidated. The present study aimed to investigate the ability of 3DG to cause hepatic insulin resistance in a cell model by assessing glucose uptake and glycogen content. In addition, the molecular signaling events, including the phosphoinositide 3-kinase (PI3K)/AKT/glucose transporter 2 (GLUT2) and PI3K/AKT/glycogen synthase kinase-3 (GSK-3) pathways, which affect hepatic insulin resistance, were further investigated using Western blot analysis. The results showed that 3DG (10-300 ng/ml) had no significant effect on HepG2 cell viability, however, the viability of the HepG2 cells decreased with exposure to concentrations of 500 and 1,000 ng/ml. Treatment with non-cytotoxic 3DG concentrations resulted in decreased uptake of glucose and glycogen content with insulin stimulation, but not under basal conditions. The insulin-induced expression of GLUT2 and p-GSK-3 were eliminated by 3DG (80 and 300 ng/ml), in addition to inhibiting the phosphorylation of downstream effectors of the insulin signaling pathway, including insulin receptor substrate 1, PI3K and AKT. In conclusion, the findings of the present study indicated that the addition of exogenous 3DG directly contributed to the induction of insulin resistance by impairing insulin signaling in the HepG2 cells, which suggested that 3DG may be involved in worsening of the diabetic condition.

Introduction

Insulin resistance, defined as a process in which the peripheral tissues, including the liver, muscle and fat, become resistant to the actions of insulin, is the primary cause of type 2 diabetes (1-3). The liver has a central role in the maintenance of plasma glucose homeostasis, and the induction of hepatic insulin resistance has been reported to be important in its involvement in the development of type 2 diabetes (4). In hepatocytes, decreased glucose uptake and glycogen levels are the hallmark of insulin resistance (5-7). In addition, insulin resistance occurs at the molecular level through dysregulation in the complex network of insulin signaling pathways (5,8).

3-Deoxyglucosone (3DG), a highly reactive dicarbonyl product, has been identified as an intermediate product of the Maillard reaction (9). 3DG has been reported to be associated with diabetes and other age-related human diseases (10-12). 3DG is elevated ~2-fold in plasma of patients with diabetes, and is involved in the development of diabetic complications (13-15). In addition to exerting its potent ability to form advanced glycation end-products, 3DG itself has certain biological activities, including suppression of enzyme activity during glucose metabolism and cell proliferation, induction of apoptosis and inactivation of glutathione reductase (16-18). Although evidence has indicated that methylglyoxal (MGO) induces insulin resistance, and impairs insulin signaling in Sprague-Dawley rats (19-21) and peripheral cells (22,23), the concentration of MGO in a variety of foods, and the levels produced from the Maillard reaction, are lower than 3DG. In addition, Kiho et al (17) reported that 3DG inhibits the activities of hexokinase and glucose-6-phosphate dehydrogenase in crude extracts from the mouse liver.

In our previous study, it was found that 3DG led to impaired plasma glucose homeostasis in healthy mice (24) and, in non-diabetic seniors, abnormal elevations in plasma 3DG were observed, which was associated with impaired glucose regulation (25). Based on this evidence, it is reasonable to hypothesize that 3DG may be important in the development of insulin resistance. However, whether exposure to 3DG...
induces insulin resistance and impairs insulin signaling pathways directly in vitro remains to be elucidated. To determine the ability of 3DG to induce insulin resistance directly, the present study determined the effect of exogenously added 3DG on glucose uptake and the contents of glycogen in the hepatocellular carcinoma, HepG2, cell line. The expression levels of insulin signaling molecules involved in insulin resistance in the hepatocytes were also investigated. The results may determine whether the addition of exogenous 3DG can directly contribute to inducing insulin resistance by impairing insulin signaling in HepG2 cells.

Materials and methods

Synthesis of 3DG. According to the method of Kato et al (26), 3-DG was synthesized from glucose. A hot solution of 6 g D-glucose, 3.3 g p-toluic acid, 6.6 ml acetic acid and 135 ml ethanol was stirred vigorously and heated in an oil bath at 90˚C for 30 min. Then, 9.9 g benzoylhydrazine was added, followed by refluxing for 6-7 h. The reaction solution was incubated at 4˚C overnight, then the resulting precipitate (3DG-bisbenzoylhydrazone) was collected by filtration through a Buchner funnel (Aladdin Industrial Corporation, Shanghai, China), and washed successively with 75 ml methanol and 75 ml diethyl ether 3 times. The product was then dried at room temperature. A solution of the 3DG-bisbenzoylhydrazone product (3 g), 90 ml ethanol, 1.5 ml acetic acid, 50 ml water and 1.6 ml freshly distilled benzaldehyde (at 40-50 mmHg, 120-130˚C) was refluxed at 90˚C for 4 h. The reaction mixture was incubated overnight at room temperature and then the filtrate was collected through a Buchner funnel and then concentrated to 70 ml using an RE-52 rotary evaporator (Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) and washed 6 times with 30 ml diethyl ether, then decolorized with 2 g activated carbon. The concentrate was evaporated down to 3 ml and 10 ml of 95% ethanol was added. The solution was then charged on Amberlite IR120B (H⁺ form) and Amberlite IR4B (OH⁻ form) ion-exchange resin columns. The final solution was evaporated to a thick syrup, then the 3DG was purified further using a flash column (silicagel 60; chloroform/methanol/water ratio, 8:0:2.0:0.1). All chemical reagents used were purchased from J&K Chemical, Ltd. (Shanghai, China) without further purification. The product was identified using infrared, 1H-nuclear magnetic resonance (NMR), 13C-NMR and mass spectroscopy (27).

HepG2 cell culture and treatment. The HepG2 hepatocellular carcinoma cells were provided by the School of Biology and Basic Medical Sciences, Soochow University (Suzhou, China), and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% (v/v) fetal bovine serum (Zhejiang Tianhang Biological Technology Co., Ltd., Huzhou, China) and antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin; Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C in a humidified atmosphere containing 5% CO2. The cells were grown to 70-80% confluence and were seeded into 96/48-well plates at a density of 5x10⁴ cells/well. The cells were then either pre-treated with 3DG (10, 80 or 300 ng/ml) in serum-free medium for 24 h, or remained untreated, with or without subsequent exposure to insulin (2.0 or 6.6 IU/ml), for the experimental groups.

Cell viability assay. Cell viability was measured using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Wuxi Zhanwang Chemical Co., Ltd., Wuxi, China). Briefly, 5x10³ cells/well were seeded in 96-well microtiter plates for 24 h at 37˚C; the cells were then pre-incubated with or without 3DG at final concentrations of 10, 50, 80, 300, 500, 800 and 1,000 ng/ml in serum-free medium supplemented with high (H-DMEM; 25 mmol/l) or low (L-DMEM; 5.6 mmol/l) glucose (Gibco; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C. The medium was subsequently removed, and 200 µl of MTT was added to a final concentration of 0.5 mg/ml. After 4 h, 150 µl dimethyl sulfoxide solution (Wuxi Zhanwang Chemical Reagent Co., Ltd., Wuxi, China) was added to solubilise the MTT formazan. The plates were placed on a mechanical shaker (Shanghai Centrifuge Institute Co., Ltd., Shanghai, China) for 10 min at room temperature, and the optical density (OD) was read at 490 nm using an enzyme-linked immunometric meter SpectraMax M2 (Molecular Devices, LLC, Sunnyvale, CA, USA), as previously described (28).

Measurement of glucose uptake. The glucose uptake was measured using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxyglucose (2-NBDG; Cayman Chemical Co., Ann Arbor, MI, USA), comparable to the study by Engelbrecht et al (29). Following treatment of the cells (5x10³ cells/well) with or without different concentrations of 3DG in L-DMEM for 24 h, the cells were washed with Krebs-ringer bicarbonate (KRB) buffer (4A Biotech Co. Ltd., Beijing, China) comprising 129 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.0 mM CaCl2, 1.2 mM MgSO4, 5.0 mM NaHCO3 and 10.0 mM HEPES. In the insulin-treated group, the cells were then exposed to 3.60 IU/ml insulin, and 100 µl serum-free KRB buffer (supplemented with 160 µM 2-NBDG) was added to the medium and incubated for 30 min at 37˚C. The cells were washed twice with KRB buffer and the radioactivity incorporated into the cells was measured using a fluorescence microplate reader (excitation/emission, 488/520; Model 680, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Subsequently, the medium was replaced with L-DMEM supplemented with 200 µl MTT, and continued to culture. After 4 h incubation at 37˚C, the OD at 490 nm was measured using a microplate reader. The error was corrected using an MTT assay.

Measurement of glycogen content. The glycogen levels in the HepG2 cells were determined using a glycogen assay kit in the presence or absence of 3.6 IU/ml insulin. Briefly, following 3DG treatment of the cells for 24 h, the medium containing L-DMEM was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology) and incubated with serum-free L-DMEM and insulin for 24 h. Subsequently, glycogen in the cells was extracted using 66% (v/v) ethanol and centrifuged for 10 min at 8,000 x g at 4˚C to remove the supernatant. The precipitates of each sample were mixed with 0.5 ml water, and the medium was subsequently added into 1 ml 0.2% (w/v) antherine reagents.
Western blot analysis. The HepG2 cells were rendered insulin resistant by treatment with 3DG, as described above. The medium containing L-DMEM and 3DG was removed from the cells, and the cells were then washed twice with ice-cold PBS and solubilised in IP lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM SDS, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM NaVO₃ and 0.5 µg/ml leupeptin (Beyotime Institute of Biotechnology). Following centrifugation at 12,000 x g at 4°C for 20 min, the supernatants were collected and used for Western blot analysis. The total protein concentrations were determined using a bicinchononic acid protein (BCA) assay kit (cat. no. P0012; Beyotime Institute of Biotechnology) comprised of BCA kit A (cat. no. P0012-1), BCA kit B (cat. no. P0012-2) and standard proteins (cat. no. P0012-3). The proteins (80-120 µg) were overloaded onto a 12% SDS-polyacrylamide gel (Thermo Fisher Scientific, Inc.), then subjected to electrophoresis and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked for 1 h in Tris-buffered saline with 1% Tween (TBST; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) containing 5% dry milk. The membranes were washed with PBS containing 0.05% Tween-20 three times, and incubated at 4°C overnight with the following antibodies (dilution, 1:1,000): Rabbit polyclonal anti-glucose transporter 2 (GLUT-2; cat. no. ab54460; Abcam, Cambridge, UK); and rabbit monoclonal anti-glycogen synthase kinase-3 (GSK-3α/β) (cat. no. 5676), rabbit polyclonal anti-p-insulin receptor substrate 1 (IRS-1; cat. no. 3070), rabbit polyclonal anti-phosphoinositide 3-kinase (PI3K)-p85 (cat. no. 4292), rabbit polyclonal anti-PI3K-p110 (cat. no. 4255) and rabbit polyclonal anti-AKT (cat. no. 9272), all purchased from Cell Signaling Technology, Inc., Danvers MA, USA. Following washing 4 times for 5 min each in TBST, the membranes were incubated with goat anti-rabbit secondary antibody [1:1,000; cat. no. GAR0072; Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China] for 2 h and visualized using an ECL detection kit (Beijing Solarbio Science & Technology Co., Ltd.). Quantification of protein bands was performed using Image-J software (version 1.42).

Statistical analysis. The results of the experiments are expressed as the mean ± standard deviation. SPSS software (version 14.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The statistical significance of differences were analyzed using Student’s t-test. P≤0.05 was considered to indicate a statistically significant difference. In the following, n describes the number of repetitions of independent experiments.

Results

3DG does not alter HepG2 cell viability at concentrations between 10 and 300 ng/ml. The HepG2 cells were exposed to different concentrations of 3DG supplemented with H-DMEM or L-DMEM for 24 h to examine the effects of 3DG on cell viability. Different concentrations of 3DG were assessed, ranging between 10 and 1,000 ng/ml, and cell viability was measured using an MTT assay. As shown in Fig. 1, compared with the untreated control, 3DG did not alter HepG2 cell viability at concentrations between 10 and 300 ng/ml, whereas 3DG exhibited a degree of inhibitory activity against the growth of the HepG2 cells at concentrations of 500 or 1,000 ng/ml.

Non-cytotoxic concentrations of 3DG induce decreased 2-NBDG uptake and glycogen content in insulin-stimulated HepG2 cells. To determine whether 3DG leads to hepatic insulin resistance, the uptake of 2-NBDG and glycogen content in the HepG2 cells were measured following treatment with non-cytotoxic concentrations (10-300 ng/ml) of 3DG for 24 h. Compared with the untreated control cells, 2-NBDG uptake was significantly increased following insulin stimulation (112.61±8.42, vs. 198.85±15.65; P<0.01). The uptake of 2-NBDG remained unchanged in the 3DG-only treated cells, however, the cells co-incubated with 80 ng/ml (162.93±20.49; P<0.05) or 300 ng/ml (151.44±11.79; P<0.01) 3DG and insulin showed significant dose-dependent decreases in 2-NBDG uptake, compared with the cells exposed to insulin only (198.85±15.65; Fig. 2A). As shown in Fig. 2B, exposure to insulin significantly increased glycogen content, compared with the control (29.743±3.712, vs. 10.151±1.102 µmol/mg, respectively). The cells treated with different concentrations of 3DG showed significant dose-dependent decreases in glycogen content, at concentrations of 80 ng/ml (22.060±1.821; P<0.05) and 300 ng/ml (16.568±1.200 µmol/mg; P<0.01), compared with the cells exposed to insulin alone (29.743±3.712 µmol/mg). However, the glycogen content remained unchanged in the 3DG-only treated cells, compared with the normal group. The effect of 3DG treatment became significant from a concentration of 80 ng/ml. Lower concentrations of 3DG did not significantly induce decreased glucose uptake or glycogen content.

Treatment with non-cytotoxic concentrations of 3DG decreases the insulin-induced expression of GLUT2 and p-GSK-3α/β in HepG2 cells. To further elucidate the action...
of 3DG, the present study evaluated the expression profile of proteins, which are important for glucose uptake and glycogen content in HepG2 cells. In the present study, treatment of the HepG2 cells with insulin alone induced a significant increase in the expression levels of GLUT2 and p-GSK-3α/β, compared with the control. Although the expression levels of GLUT2 and p-GSK-3α/β were not altered by non-cytotoxic concentrations of 3DG in the 3DG-only treated cells, significant decreases in the protein expression levels of GLUT2 and p-GSK-3α/β were observed following exposure of the cells to 3DG at concentrations of 80 and 300 ng/ml for 24 h with insulin stimulation (Fig. 3).

**Effects of non-cytotoxic concentrations of 3DG on insulin signaling in HepG2 cells: Expression and phosphorylation of IR-β, IRS-1, PI3K-p85, PI3K-p110 and AKT.** Glucose uptake and glycogen synthesis in the liver are regulated via the activation of IRS-1, PI3K and AKT (30). Exogenous 3DG treatment had no significant effect on the expression levels of IR-β and IRS-1, however, treatment of the cells with 300 ng/ml 3DG reduced the insulin-stimulated tyrosine phosphorylation of IRS-1 (Fig. 4A). To investigate the consequences of the reduced tyrosine phosphorylation of IRS-1, the expression levels of the downstream target proteins, PI3K-p85, PI3K-p110, AKT and
As shown in Fig. 4A, 3DG treatment induced significant decreases in the protein expression levels of the PI3K p85 and p110 subunits. Furthermore, 3DG led to a dose-dependent decrease in the phosphorylation of AKT, which was not accompanied by changes in the level of AKT (Fig. 4B).

Discussion

Insulin is important in the stimulation of glucose uptake, the impairment of which is a major factor responsible for insulin resistance. In addition, insulin acts by decreasing glucose production and enhancing its storage as glycogen in hepatocytes, the disorder of which is also considered to be an underlying mechanism of insulin resistance (6,7,31). In the present study, it was observed that exposure of the HepG2 cells to 3DG for 24 h at concentrations of 80 and 300 ng/ml decreased glucose uptake and glycogen content with insulin stimulation, but had no effect on glucose uptake or glycogen content in the absence of insulin (Fig. 2). In addition, decreased glucose consumption was observed in the 3DG-treated HepG2 cells following stimulation with insulin (data not shown). In agreement with a study by Kiho et al (17), these results suggested the association between 3DG and the development of insulin resistance.

In hepatocytes, the primary mechanism of insulin-stimulated glucose uptake and release in the fed and fasted states, respectively, is through GLUT2, which has a low apparent affinity for glucose (Km ~17 mmol/l) and is expressed at high concentrations...
levels in the liver (32,33). The high $K_m$ value for glucose indicates that the rate of glucose transport by GLUT2 varies with the concentrations of glucose under physiological conditions. In the present study, the expression level of GLUT2 was reduced significantly following exposure of the HepG2 cells to 3DG at 80 and 300 ng/ml for 24 h with insulin stimulation. However, in the absence of insulin, no significant decrease in the expression level of GLUT2 was observed (Fig. 3A). However, the precise role of GLUT2 in glucose transport in the liver remains to be fully elucidated. Another glucose transporter isoform, GLUT1, which shows low levels of expression in hepatocytes, has been identified to collaborate with GLUT2 to mediate glucose uptake (34). In the present study, it was shown that the protein expression of GLUT1 was reduced following stimulation with insulin and treatment of the HepG2 cells with 3DG (data not shown). Furthermore, the effect of 3DG on GLUT2 in fed and fasted states in animal models is under investigation.

GSK-3 is a serine/threonine kinase, which is associated with several diseases, including cancer, diabetes, inflammation, and Alzheimer’s disease (35,36). It has been reported that GSK-3 inactivation is involved in the positive regulation of hepatocellular carcinoma cell proliferation (37,38). In addition, insulin phosphorylates and inactivates GSK-3, leading to the activation of the glycogen synthase (GS), which is responsible for the storage of glucose in the liver (39). Therefore, the activation of GSK-3 promotes the phosphorylation and inactivation of GS, thereby decreasing glycogen synthesis. The findings in the HepG2 cells in the present study were consistent with the above. Although the expression level of p-GSK-3 was not altered by 3DG treatment under basal conditions, a reduction in the phosphorylation of GSK-3 was observed in the 3DG-treated cells stimulated with insulin (Fig. 3B). In the 3DG-only treated cells, the HepG2 cell viability was inhibited at concentrations of 500 and 1,000 ng/ml (Fig. 1), possibly due to mechanisms including the decreased expression of GLUT1 (40) and phosphorylation of GSK-3. In addition, it has been shown that 3DG can induce the apoptosis of U937 monocyte leukemia cells (41), and the results of the present study indicated that 3DG directly impaired insulin action in the HepG2 cells.

Insulin signaling pathways, for example the IR/IRS/Pi3K/AKT signaling pathway, are important in regulating the expression of GLUT2 (42-44) and GSK-3 (7,45,46). When insulin binds to IR, the intrinsic tyrosine kinase activity of IR is activated. The activated receptor then phosphorylates its own receptor and IRS, thereby dissociating IRS from IR (46). The activated receptor then phosphorylates and inactivates IRS, thereby decreasing glycogen synthesis. The findings in the HepG2 cells in the present study were consistent with the above. Although the expression level of p-GSK-3 was not altered by 3DG treatment under basal conditions, a reduction in the phosphorylation of GSK-3 was observed in the 3DG-treated cells stimulated with insulin (Fig. 3B). In the 3DG-only treated cells, the HepG2 cell viability was inhibited at concentrations of 500 and 1,000 ng/ml (Fig. 1), possibly due to mechanisms including the decreased expression of GLUT1 (40) and phosphorylation of GSK-3. In addition, it has been shown that 3DG can induce the apoptosis of U937 monocyte leukemia cells (41), and the results of the present study indicated that 3DG directly impaired insulin action in the HepG2 cells.

In the present study, the expression level of GLUT2 was reduced significantly following exposure of the HepG2 cells to 3DG at 80 and 300 ng/ml for 24 h with insulin stimulation. However, in the absence of insulin, no significant decrease in the expression level of GLUT2 was observed (Fig. 3A). However, the precise role of GLUT2 in glucose transport in the liver remains to be fully elucidated. Another glucose transporter isoform, GLUT1, which shows low levels of expression in hepatocytes, has been identified to collaborate with GLUT2 to mediate glucose uptake (34). In the present study, it was shown that the protein expression of GLUT1 was reduced following stimulation with insulin and treatment of the HepG2 cells with 3DG (data not shown). Furthermore, the effect of 3DG on GLUT2 in fed and fasted states in animal models is under investigation.

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