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ORIGINAL PAPER

GLP-1 amplifies insulin signaling by up-regulation of IRβ, IRS-1 and Glut4 in 3T3-L1 adipocytes

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Abstract Glucagon-like peptide-1 (7–36) amide (GLP-1) is an insulin secretagogue. Recently, many studies have shown GLP-1 can improve insulin resistance in peripheral tissues. In the present study, we investigated glucose uptake in 3T3-L1 adipocytes in either basal or insulin resistant state and dissected insulin signaling pathway in order to elucidate the molecular mechanisms of GLP-1 mediated improvement of insulin resistance. We found GLP-1 and its long lasting analogue, exendin 4 up-regulated basal IR, IRS-1 and Glut 4 expressions although they did not increase basal glucose uptake alone. However, GLP-1 and exendin-4 increased insulin mediated glucose uptake in intact and TNF-α treated 3T3-L1 adipocytes by up-regulation of phophorylated IRβ, IRS-1, Akt and GSK-3β. These results indicate that GLP-1 and its analogue exendin-4 can amplify insulin signaling in 3T3-L1 adipocytes by up-regulation of some crucial insulin signaling molecules.

Keywords GLP-1 · Exendin 4 · Insulin signaling · 3T3-L1 adipocytes

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Introduction

Glucagon-like peptide-1 (7–36) amide has demonstrated an obvious antidiabetic effect and widely used in the treatment of type 2 diabetes [1]. In addition to its insulinotropic effect on pancreatic beta cells, GLP-1 also increases insulin sensitivity in peripheral tissues, which suggests that GLP-1 has activity in extrapancreatic tissues and may participate in overall glucose homeostasis.

It has been demonstrated in several studies that GLP-1 increases glycogen synthesis and glucose transport by activating phosphatidylinositol-3 kinase (PI-3k) and increasing phosphorylation of protein kinase B (PKB), p70s6 kinase (p70s6k) and p42/44 MAP kinases (p44/42 MAPKs) in skeletal muscles and the liver [2–4]. GLP-1 also stimulates glucose uptake, lipid synthesis and lipolysis in adipocytes isolated from either normal rats or streptozotocin-induced diabetic rats [5].

In addition to its insulin-like effect on glucose oxidation and utilization in peripheral tissues, GLP-1 demonstrates synergistic effect with insulin in skeletal muscles and adipocyte cells [6, 7]. However GLP-1 does not have robust effect on basal glucose utilization [8]. It is still unclear through which molecular mechanism GLP-1 promotes insulin-mediated glucose utilization in adipose tissues even though it has been reported that GLP-1 could

modulate glucose transporters and PI-3k activation in the liver and skeletal muscles of normal and diabetic rats [9–11]. In the present study, we investigated glucose uptake and insulin signaling activation mediated by GLP-1 in 3T3-L1 adipocytes.

Results

Glucose uptake in 3T3-L1 adipocytes

GLP-1(7–36)-amide and Exendin-4 significantly augmented insulin-mediated glucose uptake in 3T3-L1 adipocytes by 32.3% and 34.1%, respectively, but did not increase basal glucose uptake (Fig. 1a and b). Wortmannin, a phosphatidelinositol-3 (PI-3) kinase inhibitor completely blocked this glucose uptake increase by either insulin plus GPL-1 or insulin plus exendin 4. It suggested that glucose uptake increase mediated by GLP-1 and exendin-4 may also be PI-3K activation dependent.

Insulin signaling activation

GLP-1 or exendin-4 alone only increased total IRβ, IRS-1 and Glut4 expressions instead of phosphorylated IRβ and IRS-1 in 3T3-L1 adipocytes (Fig. 2a and b), which does not lead to increase of glucose uptake. However, GLP-1 or exendin-4 up-regulated tyrosine phosphorylation of IRβ and IRS-1 in the presence of insulin (Fig. 2c). Phosphorylation of Akt and GSK-3β were also further increased by GLP-1 or exendin-4 in the presence of insulin (Fig. 2d).

GLP-1 and exendin-4 up-regulated insulin signaling in TNF-α treated 3T3-L1 adipocytes

TNF-α, as an inflammatory factor, has been known to induce insulin resistance by activation of JNK and NF-κB and suppression of insulin signaling. Glucose uptake was dramatically reduced by 49% in 3T3-L1 adipocytes treated with TNF-α for 72 h. Whereas GLP-1 and exendin 4 significantly increased glucose uptake by 22% and 21%, respectively, in TNF-α treated cells (Fig. 3a). Moreover, GLP-1 and exendin 4 up-regulated IRβ, IRS-1 and Glut4 expressions, which were suppressed by TNF-α (Fig. 3b). Phosphorylation of IRβ and IRS-1 was also up-regulated by GLP-1 or exendin 4 in TNF-α treated 3T3-L1 adipocytes in the presence of insulin (Fig. 3c). Phosphorylated Akt and GSK3β expressions were further increased by GLP-1 and exendin 4 in TNF-α treated 3T3-L1 adipocytes (Fig. 3d). Glut 4 in the plasma membrane was also increased by GLP-1 and exendin 4 (Fig. 3e).

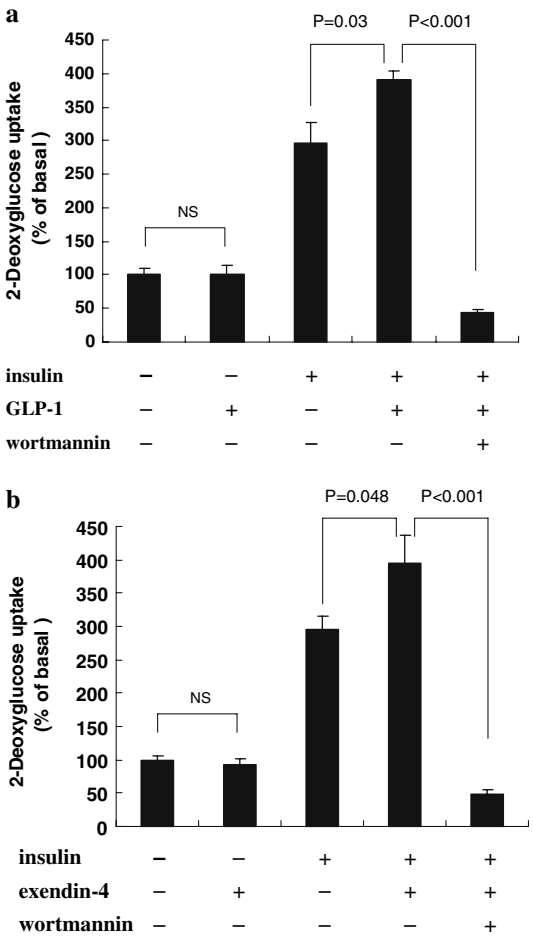

Fig. 1 2-DOG uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with GLP-1 (**a**) or exendin-4 (**b**) at 10 nmol/l for 24 h, followed with or without insulin at 10 nmol/l for 30 min. Wortmannin at 100 nM was added for 30 min. 2-DOG uptake was measured as described in "Material and methods" section. The results are presented by the folds of the vehicle control and shown as mean ± SD from one of four independent experiments. NS, no significant difference

Discussion

It has been known that GLP-1 has extensive biochemical functions, such as enhancing glucose-mediated insulin release, stimulating pancreatic β-cell growth and survival, and protecting β-cell from apoptosis [12, 13]. Additionally, GLP-1 can improve insulin resistance in peripheral tissues in patients with NIDDM and IDDM [1, 14]. A synergistic effect of GLP-1 and insulin on glucose utilization was also reported in L6 myotubes, isolated rat adipocytes and 3T3-L1 adipocytes [6–8, 11, 15]. A direct action of GLP-1 was proposed [4, 5].

However, it is still unknown about the molecular mechanism of GLP-1 in glucose utilization in peripheral tissues. In the present study, we revealed that GLP-1 and exendin-4 up-regulated basal IRβ, IRS-1 and Glut4

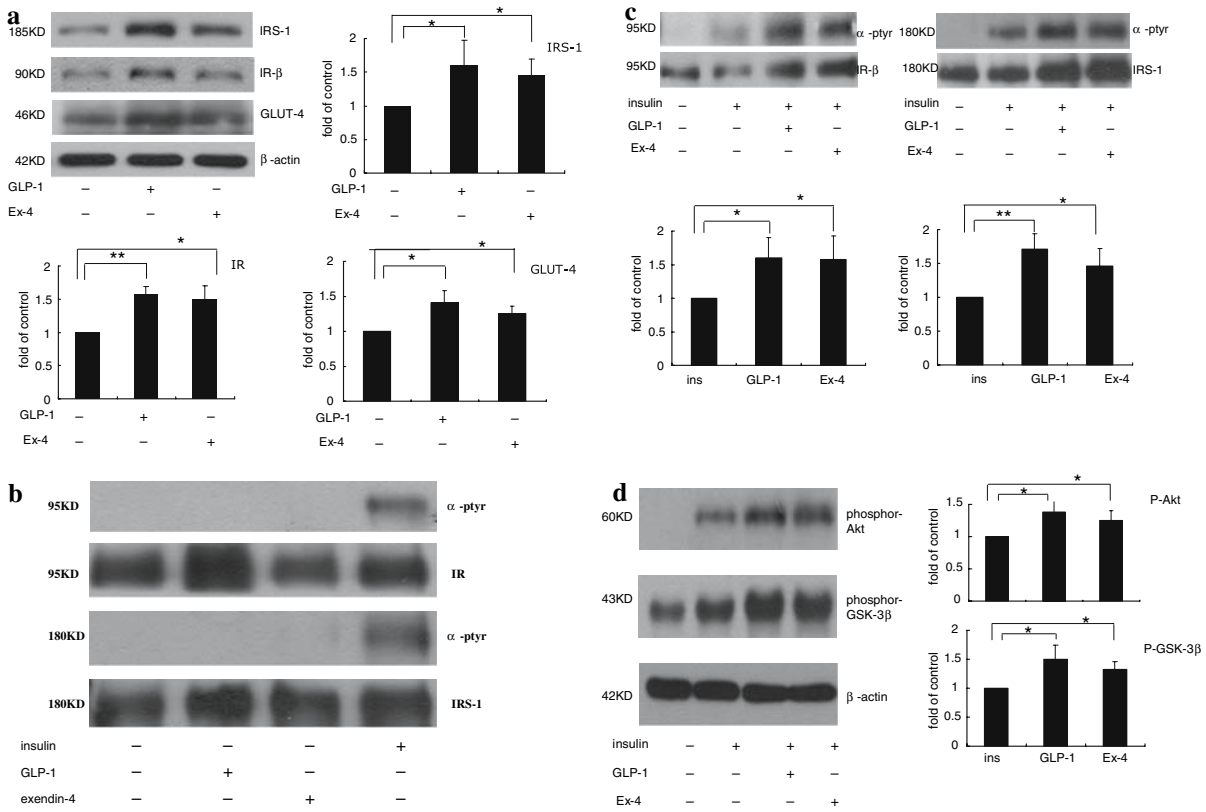

Fig. 2 Immunoblots of Insulin signaling molecules 3T3-L1 adipocytes were treated as described in Fig. 1. (**a**) Immunoblotting for IRβ, IRS-1 and Glut 4 from whole cell lysates. (**b**) and (**c**) Immunoprecipitation for phosphorylated IRβ and IRS-1. Tyrosine phosphorylated IRβ and IRS-1 were blotted with α-ptyr antibodies. (**d**) Immunoblotting for phospho-Akt (Ser473) and phospho-GSK3β. The representative immunoblots are shown from one of three independent experiments. The quantitation of the immunoblots was shown as the folds of the control. The mean and SD were derived from three different experiments. $^{*}P < 0.05$; $^{**}P < 0.01$

expression. It implies that these insulin signaling molecules are accumulated in advance to wait to be activated by phosphorylation in the presence of insulin, as we observed phosphorylated IRβ and IRS-1 were increased in 3T3-L1 adipocytes treated with GLP-1 and insulin together. PI3 kinase activation and up-regulation of phosphorylation of Akt and GSK3β were further observed.

GLP-1 modulated the glucose transporter levels in the liver, fat tissue and skeletal muscle in normal and diabetic rats [9]. GLP-1 also increased insulin-stimulated glucose uptake by up-regulation of GLUT1 and GLUT4 expression [10]. In the present study, we also found GLP-1 up-regulated GLUT4 expression, which is possibly through a translational or posttranslational regulation. This result suggested GLP-1 might have an insulinotropic effect on enhancing glucose uptake although it is unknown how GLP-1 up-regulated IRβ, IRS-1 and Glut4 expression.

It has been known that TNF-α causes insulin resistance in peripheral tissues by down-regulation of IR/IRS-1 and GLUT4 expression [16, 17] and serine 307 phosphorylation of IRS-1 [18–20]. Pioglitazone can ameliorate TNF-α induced insulin resistance by restoration of IR and IRS-1 expression [17]. In the present study, we also found that GLP-1 restored IRβ, IRS-1 and GLUT-4 expression and further increased phosphorylation of Akt and GSK-3β in TNF-α treated 3T3-L1 adipocytes. It was reported that IRS-1/2 degradation induced by TNF-α was attenuated by down-regulation of suppressor of cytokine signaling 3 (SOCS3) expression [21]. However, it needs to be further investigated how GLP-1 prevents TNF-α induced down-regulation of IRβ, IRS-1 and GLUT-4 expression.

In conclusion, GLP-1 augments insulin-mediated glucose uptake and ameliorate TNF-α mediated insulin resistance by up-regulation of insulin signaling molecules, including IRβ, IRS-1 and Glut4.

Materials and methods

Materials

Human insulin was purchased from Eli Lilly (Indianapolis, IN, USA). Bovine serum albumine (BSA) was purchased from Genebase (Shanghai, China), 3-Isobutyl-1-methylxanthine,

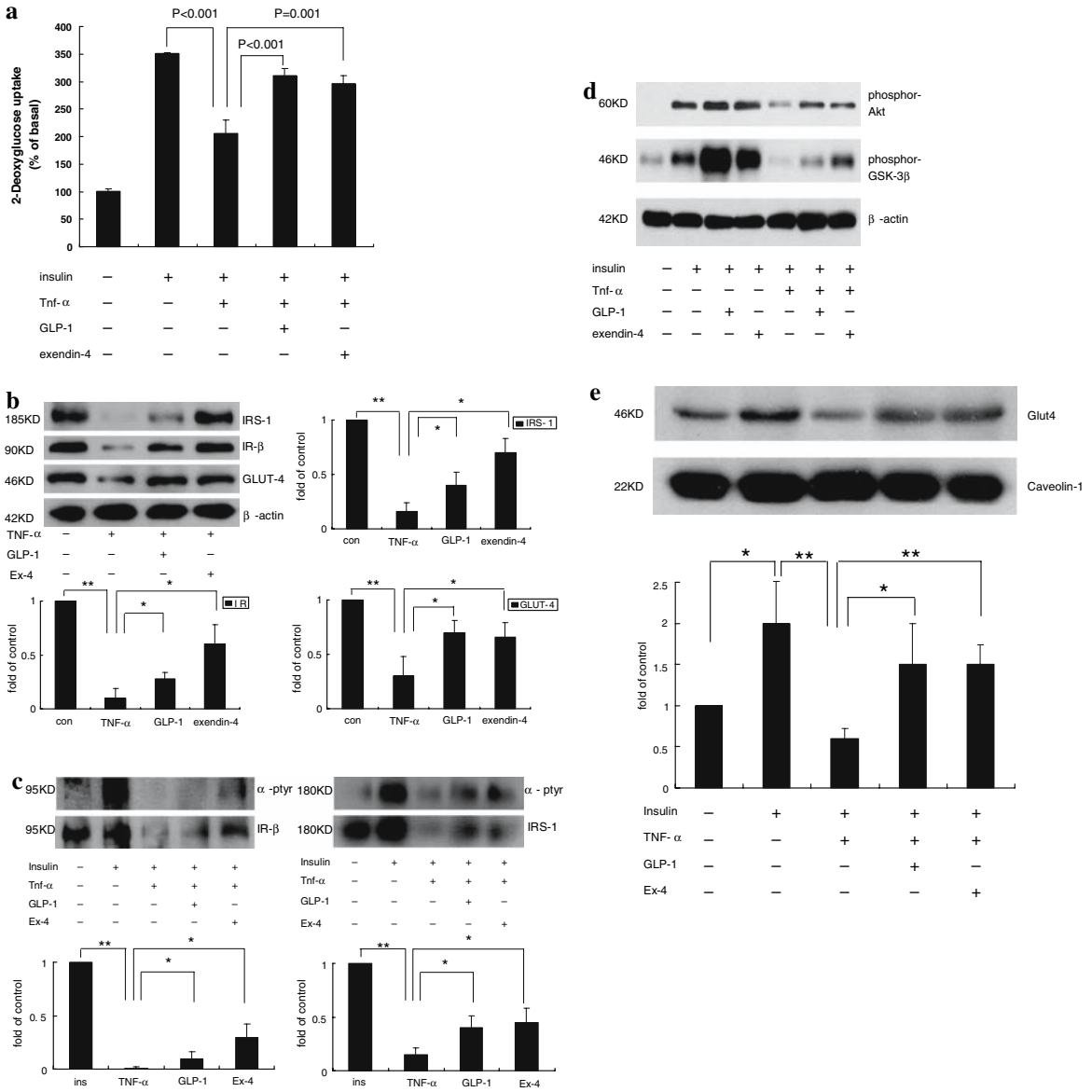

Fig. 3 2-DOG uptake and insulin signaling changes in TNF-α treated 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with TNF-α for 72 h and then with GLP-1 or exendin-4 at 10 nmol/l for the last 24 h, and finally with. Insulin at 10 nmol/l for 30 min. (**a**) 2-DOG uptake. The results are presented as the folds of the vehicle control and mean ± SD from one of four independent experiments. (**b**) Immunoblotting for IRβ, IRS-1 and GLUT4 from whole cell lysates. (**c**) Immunoprecipitation of IRβ and IRS-1. Tyrosine phosphorylated IRβ and IRS-1 were blotted with α-ptyr antibodies. (**d**) Immunoblotting for phospho-Akt (Ser473) and phospho-GSK3β. The representative immunoblots from three independent experiments are shown. (**e**) Immunoblotting for Glut4 in plasma membrane. Twenty micrograms of membrane proteins were loaded. Caveolin-1 is used as an internal control of the loading quantity. The representative immunoblots are shown from one of the three independent experiments. The quantitation of the immunoblots was shown as the folds of the control. The mean and SD were derived from three different experiments. $^{*}P < 0.05$; $^{**}P < 0.01$

dexamethasone, 2-Deoxy-D-[3H]glucose and exendin-4 from Sigma (St. Louis, MO, USA), TNFα from R&D systems (Minneapolis, MN, USA), GLP-1 from bachem (Torrence, CA, USA) and protein A-agarose from santa cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) detection system was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). Antibodies against IR, IRS-1 and phosphotyrosine (PY20) were purchased from Santa Cruz Biotechnology, Inc., anti-GLUT-4 antibody from R&D systems (Minneapolis, MN, USA). Anti-phospho-Akt (Ser473) and Akt antibodies, anti-phospho-GSK3β and GSK3 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell cultures

3T3-L1 fibroblasts were obtained from American Type Culture Collection (ATCC, USA) and cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum in an atmosphere of 7% CO2 at 37°C. Two days later when the fibroblasts had reached confluence, differentiation was induced by treating the cells with medium containing 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin for 48 h. Cells were maintained in complete DMEM every other day for the next 4–6 days until more than 90% of cells demonstrating adipocyte phenotype. The 3T3-L1 fibroblasts were used up to passage 10. Prior to each experiment, the culture medium was changed to DMEM containing 0.2% BSA. Cells were incubated with GLP-1 or exendin-4 for 24 h, respectively, with fresh medium change every 8 h containing the same mixture. In long-term TNF-α treatment assay, 3T3-L1 adipocytes were treated with 10 ng/ml TNF-α for 72 h and GLP-1 or exendin-4 for the last 24 h, respectively, otherwise it was indicated. Fresh TNF-α was added every 24 h.

2-Deoxyglucose uptake

Glucose transport was determined by measuring the uptake of [3H] 2-DOG. Cells were cultured in 24-well plates. Transport assay was initiated by washing the cells twice in a transport solution Krebs-Ringer's phosphate buffer (KRP) (20 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, and 2 mM pyruvate, pH 7.4). Cells were then incubated in the transport solution for 15 min, which contained 0.1 mM 2-DOG and 0.5 μCi [3H] 2-DOG (10 mCi/mmol). The cells were quickly washed three times with ice-cold PBS containing 10 mM glucose. The cells were lysed in 0.1 N NaOH and subsequently solubilized in scintillation fluids (Triton X-100: methylbenzene, 1:2.5) overnight. [3H] 2-DOG was measured by liquid scintillation counting. The nonspecific glucose uptake was measured by subtracting values for [3H] 2-DOG in the presence of 10 μM cytochalasin B.

Immunoprecipitation and Immunoblotting

3T3-L1 adipocytes were cultured in 6-well plates and treated with GLP-1 or exendin-4 as described above. The treated cells were lysed at 4°C with 100 μl RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1:1000 phosphatase inhibitor and 1:2,000 protease inhibitor and centrifuged at 15,000 × *g* for 15 min. For immunoprecipitation, the supernatant was incubated with IR or IRS-1 antibody at 4°C for 16 h, and with protein A-agarose beads for 2 h. The beads were washed with RIPA by five times, boiled in Laemmli sample buffer for 10 min, loaded on 6–10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h and then were incubated with appropriate antibodies. The proteins were visualized with ECL-plus kit (Amersham Pharmacia Biotech, USA).

Subcellular fractionation

The GLP-1 and exendin-4 treated 3T3-L1 adipocytes were washed with ice-cold PBS for three times and scraped in homogenization buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 255 mM sucrose, and 1× protease inhibitor mixture) at 4°C. The cell lysates were then fractionated by centrifugation [22]. Briefly, cells were homogenized in a motor-driven Teflon/glass homogenizer and centrifuged at 16,000 × *g* for 20 min (Beckman JA-17 centrifuge). The supernatant was further centrifuged at 36,000 × *g* for 5 min for high-density membrane (HDM) and then at 200,000 × *g* for 24 min for low-density membrane (LDM) fractions. The plasma membrane (PM) fraction was collected from the interface of 1.12 M sucrose cushion following centrifugation at 70,000 × *g* for 10 min. PM was then resuspended in homogenization buffer and pelleted at 200,000 × *g* for 4 min. All fractionations were resuspended in 10 mM Tris HCL buffer, pH 7.5, containing 1 mM EDTA and 1 × protease inhibitor mixture.

Statistical analysis

Quantitative values were expressed as means ± SD. Statistical significance was tested using the Student *t*-test. Values of $P < 0.05$ were considered statistically significant.

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