Treatment of type 2 diabetic db/db mice with a novel PPARγ agonist improves cardiac metabolism but not contractile function

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Carley, Andrew N., Lisa M. Semeniuk, Yakhin Shimoni, Ellen Aasum, Terje S. Larsen, Joel P. Berger, and David L. Severson. Treatment of type 2 diabetic db/db mice with a novel PPARγ agonist improves cardiac metabolism but not contractile function, Am J Physiol Endocrinol Metab 286: E449–E455, 2004. First published November 4, 2003; 10.1152/ajpendo.00329.2003.—Hearts from insulin-resistant type 2 diabetic db/db mice exhibit features of a diabetic cardiomyopathy with altered metabolism of exogenous substrates and reduced contractile performance. Therefore, the effect of chronic oral administration of 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH), a novel ligand for peroxisome proliferator-activated receptor-γ that produces insulin sensitization, to db/db mice (30 mg/kg for 6 wk) on cardiac function was assessed. COOH treatment reduced blood glucose from 27 mM in untreated db/db mice to a normal level of 10 mM. Insulin-stimulated glucose uptake was enhanced in cardiomyocytes from COOH-treated db/db hearts. Working perfused hearts from COOH-treated db/db hearts demonstrated metabolic changes with enhanced glucose oxidation and decreased palmitate oxidation. However, COOH treatment did not improve contractile performance assessed with ex vivo perfused hearts and in vivo by echocardiography. The reduced outward K⁺ currents in diabetic cardiomyocytes were still attenuated after COOH. Metabolic changes in COOH-treated db/db hearts are most likely indirect, secondary to changes in supply of exogenous substrates in vivo and insulin sensitization.

METHODS

Experimental animals. Male C57BL/KsOlaHsd-lepr db/-lepr db (db/db) and their lean, nondiabetic heterozygote littermates (db/+ ) were purchased from Harlan Laboratories (Indianapolis, IN). Mice arrived at 5–6 wk of age and were allowed to acclimatize for 1 wk. Animals were given ad libitum access to food and water and housed under a controlled lighting regimen (12:12-h light-dark cycle). Food intake and body weight were measured weekly. Glucose, insulin, total cholesterol, high-density lipoprotein cholesterol, and triglyceride concentrations were measured in blood obtained from the tail vein. Glucose was measured with a Glucotrack II (Trinity Biotech). Tissue samples were collected at the end of the experiment and stored at −80°C for subsequent analysis.}

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12:12-h light-dark cycle. All experiments were approved by the University of Calgary Animal Welfare Committee.

Treatment protocol. After the 1-wk acclimatization period, db/+ and db/db mice were randomly divided into four groups. A group each of db/+ and db/db mice received powdered chow with and without the PPARγ ligand COOH as a food admixture. COOH is an indole-acetic acid-derived PPARγ agonist (Fig. 1) from Merck (transactiva-/H9253 db/db/ of H9253 acid (COOH), a nonthiazolidinedione peroxisome proliferator-activated receptor-γ (PPARγ)-selective ligand.

Fig. 1. Structure of 2-(2-(4-phenoxy-2-propylphenoxy)ethyl)indole-5-acetic acid (COOH), a nonthiazolidinedione peroxisome proliferator-activated receptor-γ (PPARγ)-selective ligand.

NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 0.5 EDTA, containing 0.4 mM [9,10-³H]palmitate (specific activity = 2.2 × 10⁶ dpm/mmol) bound to 3% BSA and 11 nM [U-¹⁴C]glucose (specific activity = 9.1 × 10⁶ dpm/mmol). The total FA concentration in the perfusate was ~0.7 mM because the BSA used was not essentially FA free (endogenous FA content was 0.25–0.3 mM). Although oleate is the predominant FA in rodent circulation, palmitate was utilized as the exogenous substrate to be consistent with previous studies that measured palmitate oxidation by perfused mouse hearts (1, 2, 7, 8). Evans and Wang (21) have reported that oxidation rates for palmitate and oleate by perfused rat hearts were identical. The perfusate was continually gassed with 95% O₂-5% CO₂.

Hearts were perfused for 40 min (preload pressure of 15 mmHg; afterload pressure of 50 mmHg) with functional measurements and the withdrawal of perfuse samples (2.5 ml) for metabolic analysis occurring every 10 min. Coronary and aortic flows were determined through the use of graduated cylinders placed within the working heart apparatus; heart pressures were measured via a pressure transducer placed in the aortic afterload line (7) by use of CV Works (University of Calgary). The sum of aortic and coronary flows was used to determine cardiac output. The pressure signal was used for calculation of HR. Perfused hearts were allowed to beat spontaneously (spontaneous sarcomere).

The oxidation of glucose and palmitate was measured simultaneously in each heart during working heart perfusions. Perfusates contained [U-¹⁴C]glucose and [9,10-³H]palmitate, as described by Belke and colleagues (7, 8). Trapping of ¹⁴CO₂ in the perfusate was used to determine the rate of glucose oxidation; the release of ³H₂O into the perfusate was used to determine the rate of palmitate oxidation. Steady-state rates of metabolism were determined by averaging the results from perfuse samples removed at the four time points (0–10, 10–20, 20–30, 30–40 min) for each heart perfusion. At the end of the perfusion protocol, the atria were removed, and hearts were frozen and stored at ~80°C for the determination of ventricular dry weight, which was used to normalize metabolic and flow data to correct for small variations in heart size.

Preparation of isolated cardiomyocytes. Mouse ventricular cardiomyocytes were prepared essentially as described by Belke et al. (6) with some modifications. Mice (12 wk of age) were injected with 100 U of heparin intraperitoneally 30 min before administration of pentobarbital sodium (250 mg/kg ip). The heart was rapidly excised and arrested in ice-cold buffer A, consisting of (in mM): 120 NaCl, 5.4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5.6 glucose, 20 NaHCO₃, 0.6 CaCl₂, 10 2.3-butanediol monoxime, and 5 taurocholate, pH 7.5. The aorta was then cannulated, and the heart was retrogradely perfused at 37°C with buffer A gassed with 95% O₂-5% CO₂ for 4 min, followed by 10–14 min with buffer A containing 25 μM CaCl₂ and 59 μM type II collagenase (Worthington). The coronary flow rate was set at 2.5 ml/min. The free wall of the right ventricle was then removed and digested at 37°C for 5–10 min longer in presence of collagenase, 50 μM CaCl₂, and 1% (wt/vol) FA-free BSA. Dispersed myocytes were filtered through an 85-μm mesh, gently pelleted by centrifugation, and resuspended in buffer A containing 100 μM CaCl₂ and 0.6% FA-free BSA. Freshly isolated cells were then used for electrophysiological studies.

For metabolic studies, calcium concentrations were increased gradually to 1.0 mM in subsequent washings. The final viability of cardiomyocytes (percentage of rod-shaped cells that excluded trypan blue) was 75–90%, with an overall yield of 1–1.5 × 10⁶ cells/heart. To measure glucose uptake, the cells were washed once with MEM (Sigma) containing 5% fetal serum albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin and then plated in 35-mm laminin-coated tissue culture dishes. Studies were conducted 60 min after the plating to allow viable cells to stick to laminin so that nonviable cells could be removed before measurement of glucose uptake.

Glucose uptake by isolated cardiomyocytes. Glucose uptake assays were performed as described by Belke et al. (6). Plated cardiomyo-
cytes were washed twice with glucose-free DMEM (GIBCO) containing 0.2% FA-free BSA and 1.0 mM pyruvate (incubation buffer). Cells were then incubated in the absence and in the presence of insulin (10 nM) in 2.0 ml of incubation buffer for 40 min at 37°C, with 95% O2-5% CO2 gassing. Twenty microliters of a 2-deoxyglucose solution containing 130 μM of glucose-free DMEM, 15 μl of a 200 mM 2-deoxy- [14C]glucose solution, and 5 μM of 2-deoxy-[3H]glucose (ICN Biomedicals) were added to the dishes, and the incubation was continued for 20 min. The buffer was then aspirated, and the cells were washed twice with cold PBS. Cells were lysed in 300 μl of 1 M NaOH at 37°C for 20 min and then washed with 200 μl of NaOH. Thirty microliters of 12 M HCl were added to 400 μl of the lysate to normalize the pH, and radioactivity was measured. Protein assay was performed with 10 μl of the lysate by use of a Micro BCA Protein Assay Kit (Pierce Chemical). Glucose uptake is expressed as picomoles per minute per milligram of protein.

K+ current recordings. Cardiomyocytes were placed in a 1-ml chamber on the stage of an inverted microscope and perfused with a solution containing (in mM): 150 NaCl, 5.4 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES, and 5.5 glucose, brought to pH 7.4 with NaOH. The solution was bubbled with 100% O2. Currents were recorded from single cells at 20–22°C by the whole cell voltage clamp method as described previously for cardiomyocytes from mouse heart (41). The pipette solution contained (in mM): 120 K-aspartate, 30 KCl, 5 Na2ATP, 5 HEPES, 1 MgCl2, 1 CaCl2, and 10 EGTA, brought to pH 7.2 with KOH. Because currents in mouse ventricular cells are large (several nA), it was essential to minimize series resistance artificats. This was done by using low-resistance electrodes (2–4 MΩ) and by active electronic compensation (60–80%). Only well-polarized cells were used, with resting potentials of at least −65 mV.

Mouse ventricle has a variety of outward K+ currents (34). Peak outward current and the sustained current at the end of a 500-ms pulse in response to voltage steps ranging from −110 to +50 mV (holding potential of −80 mV) were measured (41). The peak outward current and the sustained current are composed of a mixture of several currents from different underlying channel proteins (25, 34). Peak and sustained currents were used for comparison between groups, since these currents determine the repolarization process of the cardiac action potential. Current densities (pA/pF) were obtained by dividing current magnitudes by cell capacitance (41).

Statistical analysis. Data are expressed as means ± SE. Differences in glucose uptake, cardiac function, and substrate metabolism were determined by ANOVA with a Student-Newman-Keuls test for pairwise comparisons. Differences between means were considered statistically significant when the P values were <0.05.

RESULTS

General features of experimental animals. At 12 wk of age, diabetic db/db mice were markedly obese and hyperglycemic relative to control db/+ heterozygotes (Table 1), as shown previously (1, 2, 7). Plasma TG and FA concentrations were not elevated in diabetic mice, in contrast to previous results showing modest elevations in serum TG and FA for male (10–14 wk) db/db mice (7). Differences in background strain may account for this difference in db/db phenotype. The present investigation used db/db mice from Harlan on a C57BL/KsOlaHsd background, whereas previous studies used Jackson db/db mice with a C57BL/KsJ background. The diabetic phenotype of db/db mice is markedly influenced by background strain (20).

Preliminary experiments showed that oral administration of COOH to db/db mice for only 10 days produced a dose-dependent decline in elevated plasma glucose and TG concentrations to near-normal levels at a dose of 30 mg/kg. In a pilot study, COOH (30 mg/kg) was administered to db/db mice (9 wk of age) daily by oral gavage. After 3 wk, glucose oxidation was increased from 0.57 ± 0.10 μmol·min⁻¹·g dry wt⁻¹ in perfused hearts (n = 8) from untreated db/db mice to 1.45 ± 0.20 μmol·min⁻¹·g dry wt⁻¹ in COOH-treated db/db hearts (n = 10). Therefore, heart metabolism was responsive to COOH administered in vivo to db/db mice. To avoid the stress of daily oral gavage, the treatment protocol was modified. 1) COOH was administered as a food admixture (30 mg/kg); 2) treatment commenced at 6 wk of age, when db/db mice exhibit modest metabolic changes but normal contractile function (2, 38); and 3) COOH was also administered to nondiabetic, db/+ control mice. The total treatment period with COOH was 6 wk (from 6 to 12 wk of age).

COOH treatment of db/db mice reduced the elevated blood glucose concentrations in untreated mice (27.2 ± 1.1 mM) to normal (10.1 ± 0.6 mM) after 6 wk (Table 1) and produced a slight but significant decrease in body weight. Plasma FA in COOH-treated db/db mice was reduced significantly, but plasma TG was unchanged. Administration of COOH to control db/+ mice did not change body weight or blood glucose.

Insulin stimulation of glucose uptake. Insulin responsiveness was tested by measuring insulin-stimulated glucose uptake by isolated cardiomyocytes. Insulin (10 nM) produced a 9.3-fold stimulation of glucose uptake by cardiomyocytes from control db/+ hearts (Fig. 2). In cardiomyocytes from untreated db/db mice, insulin-stimulated glucose uptake was reduced significantly to only 2.8-fold. Treatment of db/db mice with COOH did not change basal glucose uptake, but insulin-stimulated glucose uptake was increased significantly (Fig. 2) to a value that was no longer different from insulin-stimulated glucose uptake in control cardiomyocytes. COOH treatment of control db/+ mice produced a slight but significant (P = 0.037) increase in insulin-stimulated glucose uptake. Therefore, COOH treatment did enhance cardiac insulin responsiveness.

Metabolism of ex vivo working hearts. The metabolism of exogenous substrates (glucose and palmitate oxidation) by working perfused hearts is shown in Fig. 3. Glucose oxidation,

Table 1. General features of experimental animals

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>db/+</th>
<th>db/+ + COOH</th>
<th>db/db</th>
<th>db/db + COOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>27.5±1.2 (20)</td>
<td>30.2±0.6 (13)</td>
<td>51.2±0.9 (20)*</td>
<td>48.2±1.0 (24)*†</td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>10.0±0.4 (19)</td>
<td>9.0±0.3 (13)</td>
<td>27.2±1.1 (20)*</td>
<td>10.1±0.6 (24)*†</td>
</tr>
<tr>
<td>Plasma TG, mM</td>
<td>0.56±0.12 (4)</td>
<td>0.15 (2)</td>
<td>0.46±0.13 (4)</td>
<td>0.40±0.04 (4)</td>
</tr>
<tr>
<td>Plasma FA, mM</td>
<td>0.50±0.06 (4)</td>
<td>0.17 (2)</td>
<td>0.39±0.04 (4)</td>
<td>0.25±0.03 (4)*†</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. of mice in each group is indicated in parentheses. Data are from fed mice at 12 wk of age. TG, triacylglycerols; FA, nonesterified fatty acids. *Significantly different from db/+, †significantly different from untreated db/db.
**Cardiac Dysfunction in Diabetic Mice**

**E452**

**Fig. 2.** Glucose uptake by cardiomyocytes from control db/+ (n = 5), COOH-treated db/+ (n = 4), db/db (n = 6), and COOH-treated db/db hearts (n = 5). Glucose uptake was measured in the absence and in the presence of insulin (10 nM). *Significantly different (P < 0.05) from control untreated db/db hearts.

**Fig. 3.** Cumulative glucose (A) and palmitate oxidation (B) and steady-state rates of glucose (C) and palmitate oxidation (D) for control untreated db/+ , COOH-treated db/+ , untreated diabetic db/db, and COOH-treated db/db working hearts during 40 min of aerobic perfusion. Steady-state rates were calculated as means of the 4 measurements shown in A and B: 0–10, 10–20, 20–30, and 30–40 min. Values are means ± SE; no. of hearts in each group is indicated in parentheses. *Significantly different (P < 0.05) from control untreated db/db hearts.

**Contractile function of ex vivo working hearts.** Working perfused mouse hearts provide an experimental model to assess contractile performance under experimental conditions where preload and afterload are carefully controlled (7, 30). Perfused working hearts from db/db mice at 12 wk of age exhibited signs of contractile dysfunction. Cardiac output was reduced significantly, due entirely to a reduction in aortic flow since coronary flow was unchanged (Fig. 4). Treatment of db/db mice with COOH, despite producing normalization of glucose and palmitate oxidation (Fig. 3), did not improve either aortic flow or cardiac output (Fig. 4). Interestingly, COOH did produce a reduction in HR. Administration of COOH to control db/+ mice (n = 7) had no effect on any parameters of contractile function (HR, 322 ± 24 beats/min; cardiac output, 336 ± 26 ml·min⁻¹·g⁻¹).

**Cardiac function in vivo by echocardiography.** Systolic function in conscious mice at 12 wk of age was assessed in vivo by echocardiography, essentially as described by Semeniuk et al. (38). Diabetic db/db mice exhibited reduced contractile performance (Table 2), with decreased HR and systolic dysfunction (decreased %FS and Vcf), as observed previously (38). COOH treatment did not alter cardiac function in db/+ mice and did not improve the depressed systolic function in db/db mice.

**Outward K⁺ currents in cardiomyocytes.** Current densities for both peak and sustained outward K⁺ currents were attenuated in db/db cardiomyocytes (Table 3), as reported previously (41). COOH treatment of db/db mice did not restore currents to the normal values measured in control db/+ cardiomyocytes. In fact, administration of COOH to db/db mice produced a further decline in the sustained current (Table 3).
DISCUSSION

COOH is a nonthiazolidinedione PPARγ-selective agonist (29). Preliminary experiments established that COOH administration was effective in lowering the hyperglycemia that characterizes db/db mice, a type 2 diabetes model (20, 31). Therefore, chronic administration of COOH to both control db/+ and db/db mice for 6 wk was utilized as the main experimental model. COOH treatment completely normalized blood glucose in db/db mice (Table 1). Unexpectedly, COOH also produced a slight decrease in body weight of treated db/db mice. Laplante et al. (29) observed adipose depot-specific effects of COOH administered to rats; subcutaneous fat deposition was increased but visceral fat was reduced. Therefore, COOH-induced adipose tissue remodeling in db/db mice could account for this slight reduction in body weight. Interestingly, administration of COOH to control db/+ mice had no effect on body weight or on blood glucose (Table 1). COOH administration to db/db mice also produced a significant fall in plasma FA, although TG concentrations were unchanged (Table 1).

Ex vivo perfused hearts provide an experimental system that permits control over the supply of exogenous substrates in the perfusate. Consequently, substrate metabolism can be measured concomitantly with indexes of contractile performance (8). Perfused hearts from diabetic db/db mice show early and dramatic changes in substrate utilization (1, 2, 7). Rates of glycolysis and glucose oxidation are reduced, with enhanced rates of FA oxidation. Thus FA oxidation becomes almost the exclusive source of ATP for db/db hearts (7). Reduced carbohydrate metabolism is also a feature of perfused hearts from Zucker diabetic fatty (ZDF) rats (17), another monogenic model of type 2 diabetes (31).

The profound insulin resistance observed in db/db mice in vivo from assessments of glucoregulation (20) also extends to the heart, as insulin-stimulated glucose uptake was reduced in cardiomyocytes from db/db hearts. COOH treatment enhanced insulin-stimulated glucose uptake into db/db cardiomyocytes (Fig. 2), indicating that improvement in overall diabetic status

Table 2. Assessment of cardiac function in vivo by echocardiography with control db/+ , COOH-treated db/+, diabetic db/db, and COOH-treated db/db mice

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>db/+ (n = 12)</th>
<th>db/+ + COOH (n = 6)</th>
<th>db/db (n = 12)</th>
<th>db/db + COOH (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>707±10</td>
<td>704±10</td>
<td>654±6*</td>
<td>624±16*</td>
</tr>
<tr>
<td>SWT, mm</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
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<tr>
<td>PWT, mm</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
<td>0.7±0.02</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>1.5±0.05</td>
<td>1.6±0.10</td>
<td>2.0±0.10*</td>
<td>2.0±0.11*</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>3.6±0.06</td>
<td>3.7±0.12</td>
<td>3.7±0.05</td>
<td>3.8±0.05</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>84±3</td>
<td>79±2.5</td>
<td>87±3</td>
<td>95±4</td>
</tr>
<tr>
<td>FS, %</td>
<td>58.5±1.4</td>
<td>58.0±1.8</td>
<td>45.5±2.3*</td>
<td>46.9±2.5*</td>
</tr>
<tr>
<td>Vcf, circ/s</td>
<td>15.5±0.7</td>
<td>14.8±1.0</td>
<td>11.0±0.5*</td>
<td>9.8±0.6*</td>
</tr>
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Values are means ± SE. HR, heart rate; SWT, septal wall thickness; PWT, posterior wall thickness; LVIDd, systolic left-ventricular (LV) internal dimension; LVIDs, diastolic LV internal dimension; FS, fractional shortening; Vcf, velocity of circumferential fiber shortening; ET, ejection time. *P < 0.05 relative to db/+ control mice.

Table 3. Outward K+ currents in cardiomyocytes from control db/+ , diabetic db/db, and COOH-treated db/db mice

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>K+ Current Density (pAPF)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>db/+ (n = 12)</td>
</tr>
<tr>
<td>Peak</td>
<td>44.4±4.4</td>
</tr>
<tr>
<td>Sustained</td>
<td>22.0±1.8</td>
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</tbody>
</table>

Values are means ± SE for the number of cells in parentheses. Current densities for peak and sustained K+ currents were measured. *P < 0.05 relative to control db/+ cardiomyocytes; †P < 0.05 relative to untreated db/db cardiomyocytes.
in treated db/db mice (normalization of hyperglycemia) correlated with increased cardiac insulin responsiveness. Sidell et al. (43) reported that rosiglitazone treatment restored to normal insulin-stimulated glucose uptake by perfused hearts from insulin-resistant ZDF rats.

Administration of COOH normalized the altered pattern of metabolism in db/db hearts by increasing glucose oxidation and reducing FA oxidation (Fig. 3). Given the low level of cardiac PPARγ expression and the absence of direct effects of PPARγ ligands on either gene expression or FA oxidation in cultured cardiomyocytes (24), the COOH-induced changes in perfused heart metabolism will most likely be an indirect mechanism, secondary to altered substrate supply to the heart in vivo (glucose- and FA-lowering actions). The observation that COOH treatment of control db/+ mice had no effect on cardiac glucose and palmitate oxidation (Fig. 3) is consistent with this conclusion. Increased cardiac insulin responsiveness in vivo may also be a contributory mechanism for metabolic changes. A key objective of future studies will be to determine the molecular mechanism(s) responsible for the alteration in cardiac metabolism induced by COOH treatment of db/db mice.

Hearts from db/db mice also exhibit contractile dysfunction, evident from studies with ex vivo perfused hearts (1, 2, 7) and in vivo assessment by echocardiography (38). A number of factors could contribute to the pathogenesis of contractile dysfunction (diabetic cardiomyopathy) in db/db hearts. First, sustained hyperglycemia in vivo could increase nonenzymatic glycation of interstitial proteins, which will produce myocardial stiffness and impaired cardiac contractility (3, 16), along with other glucotoxicity mechanisms (32, 36, 42). Second, altered metabolism of exogenous substrates in db/db hearts could contribute to contractile dysfunction, an example of metabolic maladaptation (45). Reduced glycolytic ATP generation may impair the function of key ion channels and pumps (41) reported that rosiglitazone treatment restored to normal insulin-stimulated glucose uptake by perfused hearts from insulin-resistant ZDF rats.

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