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Reconstituted high-density lipoprotein infusion modulates fatty acid metabolism in patients with type 2 diabetes mellitus

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Abstract We recently demonstrated that reconstituted high-density lipoprotein (rHDL) modulates glucose metabolism in humans via both AMP-activated protein kinase (AMPK) in muscle and by increasing plasma insulin. Given the key roles of both AMPK and insulin in fatty acid metabolism, the current study investigated the effect of rHDL infusion on fatty acid oxidation and lipolysis. Thirteen patients with type 2 diabetes received separate infusions of rHDL and placebo in a randomized, cross-over study. Fatty acid metabolism was assessed using steady-state tracer methodology, and plasma lipids were measured by mass spectrometry (lipidomics). In vitro studies were undertaken in 3T3-L1 adipocytes. rHDL infusion inhibited fasting-induced lipolysis ($P = 0.03$), fatty acid oxidation ($P < 0.01$), and circulating glycerol ($P = 0.04$). In vitro, HDL inhibited adipocyte lipolysis in part via activation of AMPK, providing a possible mechanistic link for the apparent reductions in lipolysis observed in vivo. In contrast, circulating NEFA increased after rHDL infusion ($P < 0.01$). Lipidomic analyses implicated phospholipase hydrolysis of rHDL-associated phosphatidylcholine as the cause, rather than lipolysis of endogenous fat stores. rHDL infusion inhibits fasting-induced lipolysis and oxidation in patients with type 2 diabetes, potentially through both AMPK activation in adipose tissue and elevation of plasma insulin. The phospholipid component of rHDL also has the potentially undesirable effect of increasing circulating NEFA.—Drew, B. G., A. L. Carey, A. K. Natoli, M. F. Formosa, D. Vizi, M. Reddy-Luthmoodoo, J. M. Weir, C. K. Barlow, G. van Hall, P. J. Meikle, S. J. Duffy, and B. A. Kingwell. **Reconstituted high-density lipoprotein infusion modulates fatty acid metabolism in patients with type 2 diabetes mellitus.** ***J. Lipid. Res.*** **2011.** 52: **572–581.**

Supplementary key words rHDL • lipolysis • adipose

High-density lipoprotein (HDL) elevation is a current, intense area of focus in cardiovascular therapeutics. The rationale behind this effort is centered on both the inverse relationship between HDL and cardiovascular disease in humans (1), and underpinning mechanistic data demonstrating a spectrum of HDL-mediated anti-atherosclerotic actions beyond the benefits of reverse cholesterol transport alone (2–6). Among these is the recent evidence that HDL may influence glucose metabolism via mechanisms including elevation in skeletal muscle glucose uptake via the AMP-activated protein kinase (AMPK) pathway (7, 8) and stimulation of pancreatic insulin secretion (7, 9). The important role of both AMPK and insulin in regulation of lipolysis and fat oxidation raises the possibility that HDL may also modulate fatty acid metabolism via these mechanisms.

Dyslipidaemia associated with Western lifestyle is characterized by reduced circulating HDL levels and elevated low-density lipoprotein (LDL) and nonesterified (free) fatty acids (NEFA) (10). Elevated blood NEFA has been strongly associated with the pathogenesis of insulin resistance in peripheral tissues, including skeletal muscle, liver, and adipose tissue (11, 12). Both lipolysis and fatty acid oxidation rates are key determinants of plasma NEFA concentration. The recent finding that HDL activates AMPK (2, 7, 8, 13), an enzyme which inhibits lipolysis in adipose tissue (14–17) and promotes skeletal muscle fat oxidation (18–20), suggests that low HDL may exacerbate plasma NEFA elevation and insulin resistance and contribute to the pathophysiology of type 2 diabetes. Conversely,

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; apo, apolipoprotein; CE, cholesterol ester; Cer, ceramide; CETP, cholesteryl ester transfer protein; DAG, diacylglycerol; rHDL, reconstituted high-density lipoprotein; ISO, isoproteronol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PF, phenformin; TG, triglyceride.

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HDL-raising agents currently in development to combat atherosclerotic cardiovascular disease could potentially reduce plasma NEFA and protect against type 2 diabetes. These postulates may contribute to a mechanistic basis for the inverse epidemiological associations between HDL and metabolic disease (21–23).

The potential interplay between HDL and NEFA is made more complex by a suite of studies showing that HDL and its receptor ABCA1 modulate pancreatic β-cell insulin secretion (7, 9, 24–26). Insulin promotes a shift away from utilization of fat as an energy substrate; thus, in the context of HDL elevation, insulin would be expected to synergize with AMPK activation to inhibit lipolysis in adipose tissue but would oppose AMPK-mediated fat oxidation in muscle. The net whole body effects of HDL elevation with regard to fatty acid metabolism and plasma NEFA concentrations are unknown and can only be investigated using an in vivo approach.

The current study aimed to determine the effects of acute HDL elevation on fat oxidation and lipolysis in patients with type 2 diabetes. The clinical relevance relates to both understanding the role of low HDL in the etiology of insulin resistance and the potential application of HDL-raising therapies, including rHDL, in the treatment of insulin resistance and type 2 diabetes.

METHODS

Human rHDL infusion study

The current investigation concerning fatty acid metabolism was performed in parallel with previous studies from our laboratory; thus, patient characteristics and study design have been previously reported (7, 27, 28). Briefly, 13 patients with type 2 diabetes mellitus participated in the study and received 80 mg/kg (lyophilised rHDL is reconstituted with water for injection and is infused based on protein content of the rHDL particle) (CSL Behring AG, Bern, Switzerland) over 4 h and saline placebo on separate occasions separated by at least two weeks in a double-blind crossover study.

rHDL constituents and preparation

rHDL consists of apolipoprotein AI (apoAI) isolated from pooled human plasma and phosphatidylcholine (PC) from soy bean and was prepared as previously described (7, 28). rHDL undergoes rapid remodeling and/or interaction with endogenous HDL (28) and has been previously shown to produce biological responses analogous to native HDL (29). The preparation did not contain any other proteins (leptin, insulin, adiponectin) likely to induce a metabolic response (data not shown).

Palmitate tracer preparation and infusion

Stably ^{13}C-labeled palmitate (U-^{13}C-palmitate) (Cambridge Isotope Laboratories) was dissolved and bound to 20% human albumin (Australian Red Cross) and delivered according to previously validated protocols (30–33). $NaH^{13}CO_3$ (1.5 μmol/kg; Cambridge Isotope Laboratories) was administered as a bolus followed by a constant palmitate infusion (0.015 μmol/kg/min) for the duration of the study. A 2 hr equilibration period was implemented to achieve steady-state tracer concentrations prior to commencement of the rHDL/placebo infusion (30–33).

Gas exchange and collection of expired air

Breath by breath VO_2 and VCO_2 were measured for 2 min every 30 min during the infusions (CosMed Gas Analyzer using Quark B^2; Rome, Italy). Expired air was collected into a 50 l Douglas Bag for 5 min and sampled into glass rubber-stoppered blood serum (SST) vacutainers.

Plasma U-^{13}C-palmitate enrichment and flux

Plasma palmitate tracer concentration was determined by gas chromatography (GC) (Autosystem XL, Perkin Elmer) and plasma [U-^{13}C]palmitate enrichment was determined by GC-combustion isotope ratio mass spectrometry (GC-C-IRMS) as previously described (30).

Palmitate flux was calculated using the steady-state equation described by Steele et al. and Wolfe et al. (34, 35).

$$R_a(\text{palmitate}) = \frac{\text{F} - pV \times [(C_1 + C_2)/2] \times [(E_2 - E_1)/(t_2 - t_1)]}{[(E_1 + E_2)/2]}$$

where F = tracer infusion rate, pV = volume of distribution for palmitate (pre-determined at 0.04), C = fasting plasma palmitate concentration at time 1 and time 2, E = tracer (labeled palmitate) to tracee (endogenous palmitate) ratio (TTR) at time 1 and time 2, and t = time.

R_d of palmitate (μmol/min/kg) is a function of R_a minus the change in total plasma palmitate between two time points (t_1 and t_2), and as such the equation is as follows:

$$R_d(\text{palmitate}) = R_a(\text{palmitate}) - \frac{pV \times (C_2 - C_1)}{(t_2 - t_1)}$$

Palmitate oxidation rate

Palmitate oxidation was calculated by determining the amount of expired $^{13}CO_2$ generated from the catabolism of labeled palmitate tracer. Oxidation rates are a function of the plasma enrichments, acetate correction, and VCO_2 using the equation below as previously described (31, 36).

$$\text{Palmitate Oxidation Rate} = \frac{(E_{CO2} \times V_{CO2})/16}{E \times a_r}$$

where E(CO2) = labelled CO2:unlabelled CO2 ratio in expired breath, V_{CO2} = volume of carbon dioxide expired per breath (μmol/min), 16 = the number of carbon atoms present in one palmitate molecule, and E = plasma tracer and a(r) = acetate correction factor.

Plasma lipid analysis

Plasma was collected and analyzed for HDL, LDL, total cholesterol, apoAI, apoB, and insulin as previously described (7). Plasma NEFA was measured using the WAKO NEFA kit (WAKO, VA) per the manufacturer's instructions. Plasma triglycerides were measured using the WAKO TrigA kit (WAKO, Japan) per the manufacturer's instructions. Plasma glycerol was measured using an EnzyChrom Glycerol Assay Kit per the manufacturer's instructions (BioAssay Systems, CA).

Lipidomic analysis (HPLC and mass spectrometry)

Before analysis, lipids were extracted from plasma (10 μl) with chloroform/methanol (2:1; 20 vol) following the addition of internal standards: 100 pmol each of ceramide 17:0 (Matreya Inc.,

Pleasant Gap, PA), PC (13:0/13:0) and lysophosphatidylcholine (LPC) 13:0; 1000 pmol each of free cholesterol-d_7 (Avanti Polar Lipids, Alabaster, AL) and cholesterol ester 18:0-d_6 (CDN Isotopes, Pointe-Claire, Quebec, Canada); and 100 pmol triglyceride (TG) 17:0/17:0/17:0 and 200 pmol diacylglycerol (DAG) 15:0/15:0 (Sigma-Aldrich, St Louis, MO). Extracts were centrifuged (13,000 *g*, 10 min), and the supernatant was dried under nitrogen at 40°C. Lipids were redissolved in 100 μl water saturated BuOH/MeOH (1:1) containing 10 mM NH_4COOH. Quantitation was performed by liquid chromatography electrospray ionization-tandem mass spectrometry using an Applied Biosystems 4000 QTRAP. Liquid chromatography was performed on a Zorbax C18, 1.8 μm, 50 × 2.1 mm column at 300 μl/min using the following gradient conditions: 0–100% B over 8.0 min, 2.5 min at 100% B, a return to 0% B over 0.5 min, then 3.0 min at 0% B prior to the next injection. DAG was separated using the same solvent system with an isocratic flow (100 μl/min) of 85% B. Solvents A and B consisted of tetrahydrofuran: methanol:water in the ratios (30:20:50) and (75:20:5), respectively, both containing 10 mM NH_4COOH. Quantification of individual lipid species was performed using scheduled multiple-reaction monitoring (MRM) in positive ion mode (37, 38). Lipid concentrations were calculated by relating the peak area of each species to the peak area of the corresponding internal standard. Cholesterol ester (CE) species were corrected for response factors determined for each species. Total measured lipids of each class were calculated by summing the individual lipid species.

NEFA was extracted using a scaled-down Dole extraction (39), followed by derivatization to its corresponding N,N,N-trimethylethylenediamine (TMEN) iodide salt by a method similar to that described by Johnson for the trimethylaminoethylester iodide salt (40, 41). Briefly, extracted fatty acids (10 μl plasma) containing 250 pmol of FA 17:0-d3 (CDN Isotopes) were treated successively with thionylchloride (20 μl, 0.2 M in dichloromethane, 10 min RT) N,N-dimethylethyenediamine (60 μl, 10 min RT), and methyliodide (60 μl, 50% v/v in methanol, 2 min RT) with each reagent/solvent removed under a stream of nitrogen prior to the addition of the next. The TMAA-fatty acids were reconstituted in 100 μl ethanol, and samples (1 μl) were injected into the Applied Biosystems 4000 QTRAP using a 200 μl/min flow of solvent B. Quantification was performed using MRM for the loss of 59 Da corresponding to the elimination of trimethylamine. Each scan was acquired over a 1 min period, and the peak areas were normalized to the internal standard (FA 17:0 (d3)) prior to the adjustment for the relative response factor of each fatty acid.

3T3-L1 adipocyte culture and cellular lipolysis

Pre-adipocytes were cultured in normal growth media (α-MEM containing 10% serum), differentiated using the standard DMI cocktail for four days and encouraged to lipid load in the presence of 10 nM insulin. Cells were treated with HDL (50 μg/ml), isoproteronol (10 μM), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (2 mM), or phenformin (1 mM) in media containing 0.1% fatty acid-free BSA for 4 hrs. Following this, media was collected and analyzed for NEFA and glycerol release as described above. Cells were harvested and lysates were subjected to Western blot analysis.

Western blotting

Cells were harvested and the phosphorylation of signaling molecules AMP-activated protein kinase (AMPK) and the key downstream modulator of fatty acid β oxidation, acetyl-CoA carboxylase (ACC), were determined by Western blot as previously described (7).

Statistics

Normally distributed data were compared by *t*-tests or repeated measures ANOVA with least significant difference post hoc tests used to compare individual means as appropriate. The order of the rHDL and placebo infusions was included as a between-subjects variable in the analysis of the clinical studies. Non-normally distributed data were compared by Mann-Whitney Rank Sum tests or Kruscal-Wallis one-way ANOVA on ranks with Dunn's post hoc tests to compare individual means as appropriate. Results are expressed as means ± SEM unless otherwise indicated. All analyses were conducted using SPSS (version 16). Cell culture data represent a minimum of three separate experiments performed in triplicate. $P < 0.05$ was considered significant.

RESULTS

The characteristics and demographics of the patients in these studies have been detailed previously (7, 27, 28).

rHDL infusion and conventional plasma lipids

Infusion of reconstituted HDL (rHDL) resulted in significant ($P < 0.001$) elevations in both HDL-cholesterol and apoAI protein levels (1.33-fold ± 0.43 and 2.41-fold ± 0.15, respectively; **Table 1**) compared with placebo infusion, as previously described (7). LDL-cholesterol was reduced ($P = 0.03$) with rHDL infusion, but there was no change in apoB protein levels (Table 1). Plasma insulin was elevated by rHDL by 3.4 ± 10.0 pmol/l, while the placebo group fell by 19.2 ± 7.4 pmol/l ($P = 0.034$, rHDL versus placebo at 4 hr; Table1 and Ref. 7).

TABLE 1. Plasma lipid and lipoprotein levels before and after rHDL and placebo infusion

	Placebo			rHDL			
	Before	After	*P*	Before	After	*P*	*P*[a]
apo-AI (mg/dl)	74.8 ± 3.25	76.8 ± 5.5	NS	72.1 ± 3.1	166.8 ± 10.2	<0.001	<0.001
apo-B (mg/dl)	69.6 ± 03.6	66.0 ± 2.8	NS	67.3 ± 2.4	68.5 ± 3.2	NS	NS
HDL-C (mmol/l)	0.95 ± 0.05	0.86 ± 0.05	NS	0.96 ± 0.04	1.30 ± 0.07	<0.001	<0.001
TC (mmol/l)	4.30 ± 0.23	3.97 ± 0.21	NS	4.39 ± 0.22	4.77 ± 0.2	NS	0.01
LDL-C (mmol/l)	3.03 ± 0.24	2.65 ± 0.22	NS	3.07 ± 0.27	1.99 ± 0.19	<0.01	0.03
Insulin (pmol/l)	68.7 ± 11.2	53.6 ± 6.8	0.09	68.4 ± 7.8	70.2 ± 10.7	NS	0.03

N = 13. apo-AI, apolipoprotein-AI; apo-B, apolipoprotein-B; HDL-C, HDL-cholesterol; rHDL, reconstituted HDL; LDL-C, LDL-cholesterol; NS, not significant; TC, total cholesterol.

[a]Placebo versus rHDL "after" values.

rHDL infusion inhibited fasting-induced fatty acid flux and oxidation

During the placebo infusion, rates of palmitate appearance (R_a) and disappearance (R_d) in the plasma significantly ($P < 0.05$) increased (% increase at end of infusion: $R_a = 44 \pm 8\%$, $R_d = 38 \pm 7\%$, $P < 0.001$; **Fig. 1A**). This was associated with an increase in palmitate oxidation rate of 50 ± 9% ($P < 0.001$ from baseline; Fig. 1B). In contrast, infusion of rHDL did not substantially change plasma palmitate turnover (% increase at end of infusion: $R_a = 16 \pm 9\%$, $R_d = 9 \pm 7\%$, $P = 0.03$ and $P = 0.11$ from baseline, respectively; Fig. 1A) or oxidation rate (% increase at end of infusion: rHDL = 23 ± 9%, $P < 0.01$ from baseline; Fig. 1B) from baseline, indicating an inhibition of fasting-induced lipolysis and fatty acid oxidation compared with placebo.

Consistent with increased fat oxidation, plasma glycerol significantly increased throughout the placebo infusion; however, this was completely inhibited during rHDL infusion (% change at end of infusion: placebo = 43 ± 19.5%, rHDL = −3 ± 10.8%, $P = 0.03$ and $P = 0.78$, respectively; **Fig. 2A**). A corresponding increase in plasma NEFA during placebo (26 ± 13%, $P = 0.06$) was also observed with a reduction in triglycerides (−11 ± 4.6%, $P = 0.03$). Despite glycerol and tracer data suggesting an inhibition of lipolysis by rHDL, plasma NEFA increased significantly after rHDL compared with placebo (% increase at end of infusion: placebo = 26 ± 13%, rHDL = 61 ± 18.7%, $P = 0.06$ and $P < 0.01$, respectively; Fig. 2B). This occurred in association with a significant rHDL-induced increase in plasma triglycerides (% change at end of infusion: placebo = −11 ± 4.6%, rHDL = 49 ± 10.6%, $P = 0.03$ and $P = 0.001$, respectively; Fig. 2C).

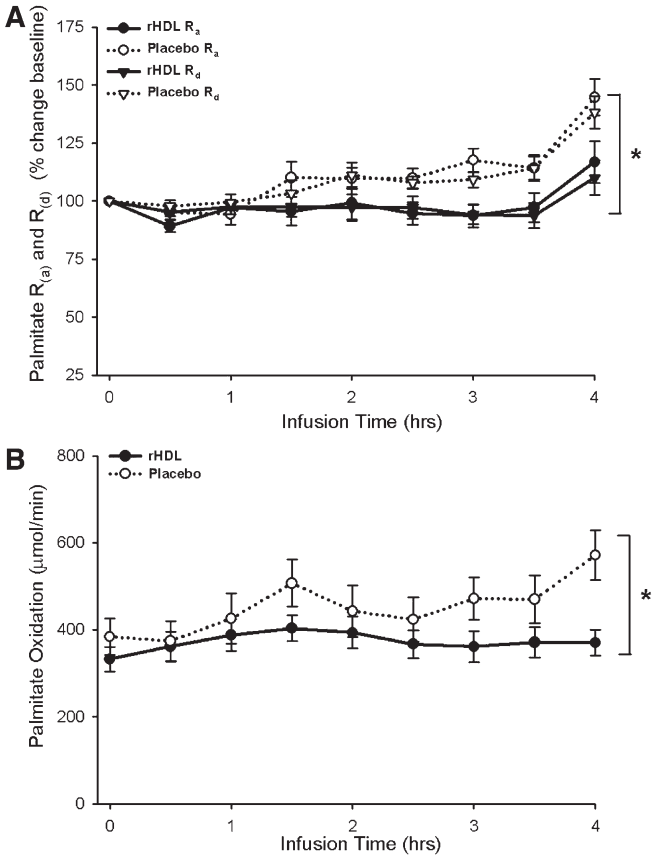

Fig. 1. Fatty acid (palmitate) tracer kinetics during rHDL and placebo infusion. A: Percent change (compared with baseline, 0 hr; actual baseline values: rHDL R_a, 2.87 ± 0.17 μM; Placebo R_a, 2.66 ± 0.12 μM; rHDL R_d, 2.96 ± 0.21 μM; Placebo R_d, 2.67 ± 0.15 μM) in rates of plasma palmitate tracer appearance (R_a, circles) and disappearance (R_d, triangles) during 4 hr rHDL (solid lines) and placebo (dotted lines) infusion. B: Rates of palmitate tracer oxidation (μmol/min) during 4 hr rHDL (solid lines) and placebo (dotted lines) infusion. *$P < 0.05$ between groups as measured by repeated measures-ANOVA, n = 13. rHDL, reconstituted HDL.

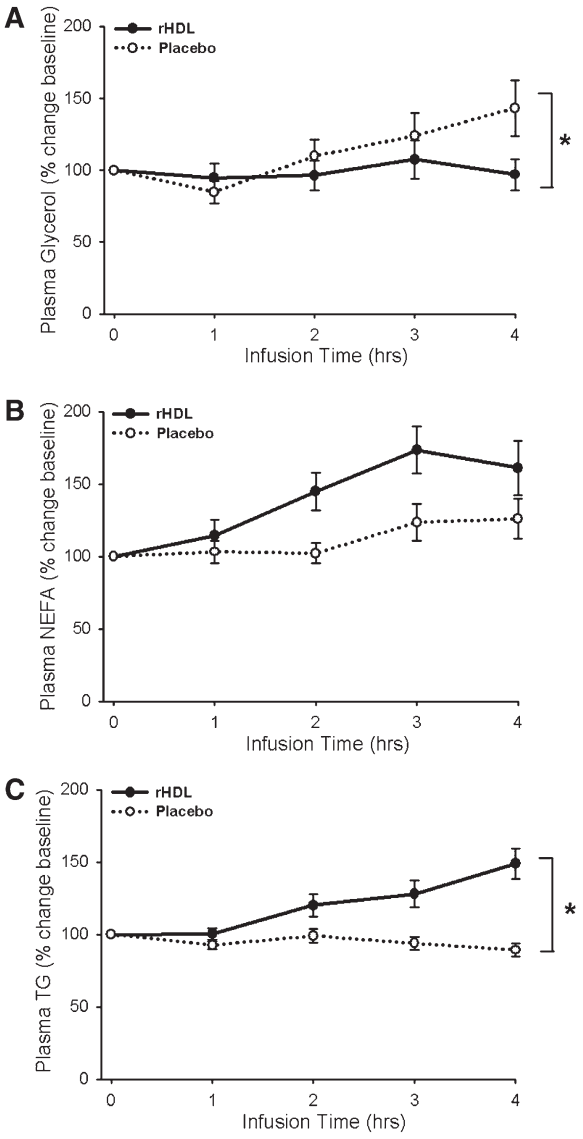

Fig. 2. Measures of basic lipolysis markers in plasma of patients during rHDL and placebo infusion. Percent change (compared with baseline, 0 hr) in levels of plasma (A) glycerol (actual baseline values; rHDL, 46 ± 11 μM; Placebo, 44 ± 9 μM); (B) NEFA (actual baseline values; rHDL, 388 ± 76 μM; Placebo, 384 ± 39 μM); and (C) TG (actual baseline values; rHDL, 1.86 ± 0.36 mM; Placebo, 1.56 ± 0.23 mM) during 4 hr rHDL (solid lines) and placebo (dotted lines) infusion. *$P < 0.05$ between groups as measured by repeated measures-ANOVA, n = 13. rHDL, reconstituted HDL; TG, triglyceride.

HDL activated the AMPK pathway and inhibited lipolysis in cultured adipocytes

As expected, the AMPK agonists AICAR and phenformin induced a significant ($P < 0.05$) increase in the phosphorylation of AMPK (1.9-fold ± 0.5 and 2.1-fold ± 0.4, respectively; **Fig. 3A**) and its downstream target ACC (1.7-fold ± 0.1 and 1.9-fold ± 0.2, respectively) in 3T3-L1 cells (Fig. 3B). HDL stimulated a modest, nonsignificant increase in AMPK phosphorylation (1.2-fold ± 0.2, Fig. 3A) but a highly significant ($P < 0.001$) increase in ACC phosphorylation (1.4-fold ± 0.1; Fig. 3B), suggesting that HDL activated the AMPK pathway in 3T3-L1 adipocytes.

Furthermore, AICAR and phenformin significantly ($P < 0.01$) reduced the amount of both NEFA (−32 ± 3.3% and −43 ± 4.7%, respectively) and glycerol (−33 ± 1.7% and −36 ± 6.9%, respectively) released into the media (Fig. 3C, D), whereas isoproterenol significantly ($P < 0.05$) increased both glycerol and NEFA release from 3T3-L1 cells (173 ± 13% and 218 ± 37.6%, respectively) (Fig. 3C, D). Treatment of 3T3-L1 cells with HDL significantly ($P < 0.05$) reduced both glycerol and NEFA release (−20 ± 4.2% and −23 ± 1.6% respectively; Fig. 3C, D), consistent with inhibition of lipolysis.

Lipidomics analysis

The concentration of individual families of lipid species before (pre) and after (post) both infusions for each group, including DAG, ceramide (Cer), free cholesterol, CE, PC, LPC, and NEFA, are shown in **Fig. 4**. All were unchanged after the placebo infusion, and there was no effect of rHDL on total DAG or Cer. Small, but significant increases were seen in free cholesterol and cholesterol esters after rHDL infusion. There were significant increases in total PC (1563 ± 96 μmol/l versus 2944 ± 117 μmol/l for placebo and rHDL, respectively); LPC (221 ± 19 μmol/l versus 740 ± 37 μmol/l

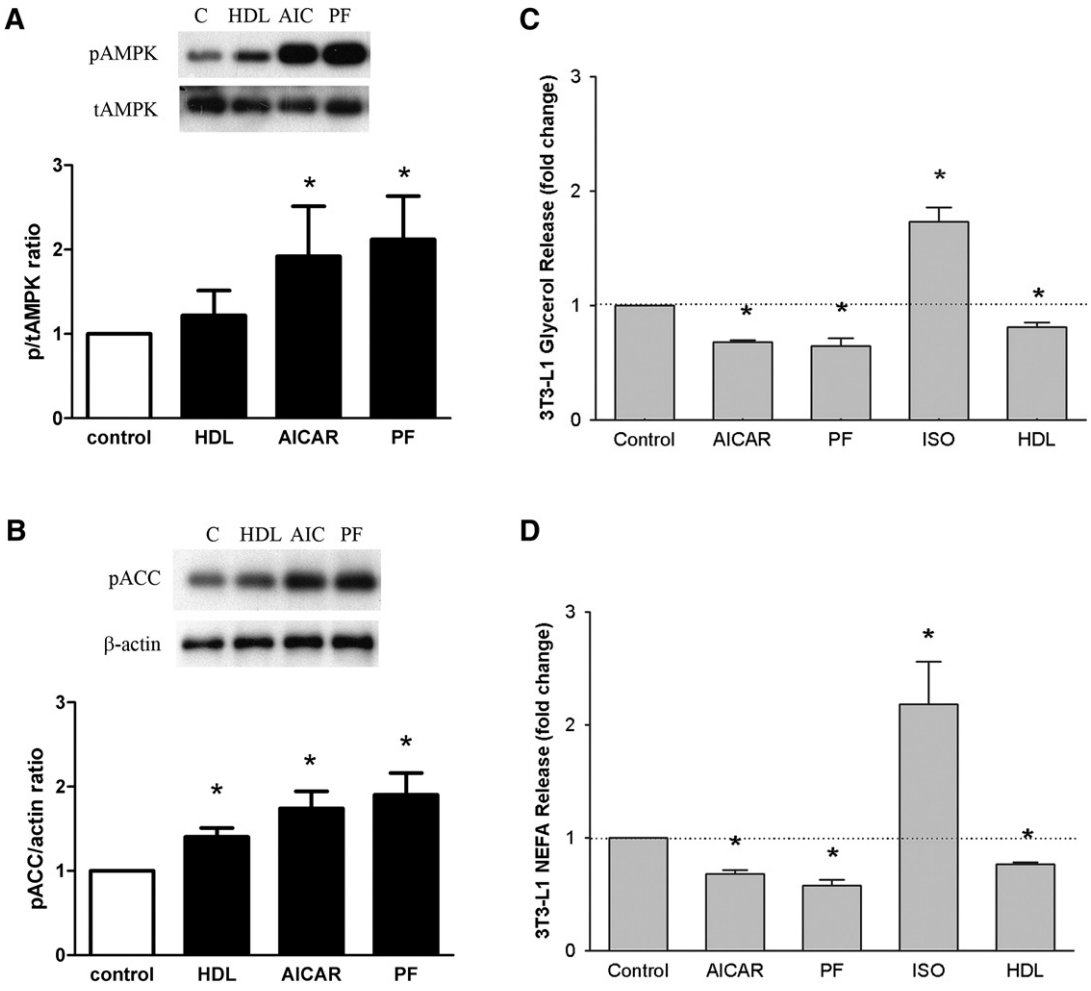

Fig. 3. In vitro AMPK signaling and lipolysis after HDL treatment in 3T3-L1 adipocytes. Differentiated and lipid-loaded 3T3-L1 adipocytes were treated for 4 hrs with AICAR (2 mM), PF (1 mM), ISO (10 μM), and HDL (50 μg/ml) before cells and media were harvested. Cell lysates were analyzed for (A) AMPK phosphorylation and protein level and (B) its downstream target ACC phosphorylation and β-actin protein expression. Media was analyzed for the amount of (C) glycerol and (D) NEFA released by adipocytes into the media, expressed as fold change from control (amount of release under basal/control conditions; glycerol, 223 ± 32 μM and NEFA 310 ± 44 μM). *$P < 0.05$ from control as measured by one-way ANOVA, n = 5. AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; ISO, isoproteronol; PF, phenformin.

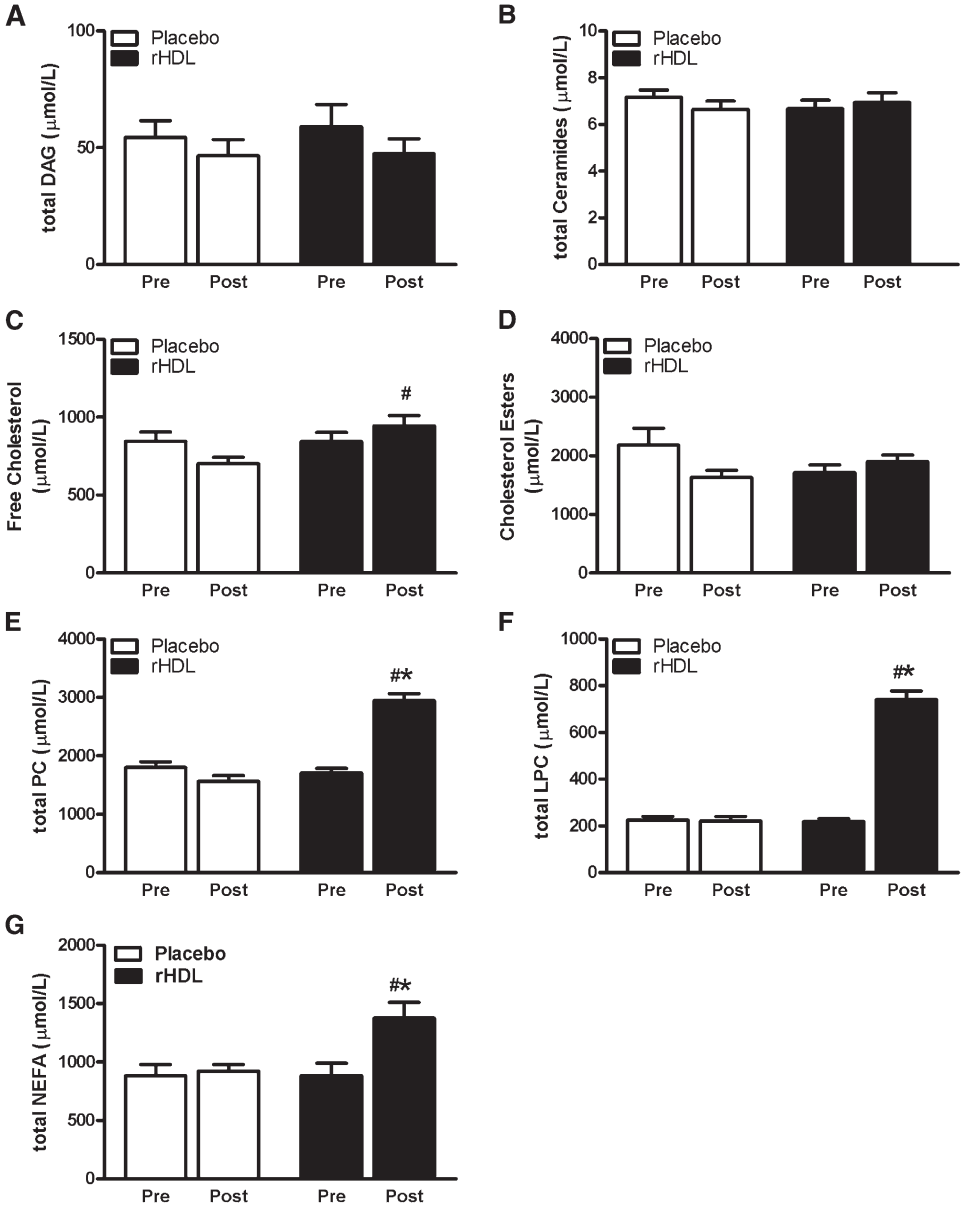

Fig. 4. Plasma lipidomics analysis for total amounts of selected plasma lipids pre- and post-rHDL and placebo infusions. Total amounts of plasma (A) DAG, (B) ceramides, (C) cholesterol, (D) cholesterol ester, (E) PC, (F) LPC, and (G) total NEFA in patients before and after infusion of rHDL (black bars) and placebo (white bars). $^{\#}P < 0.05$ between placebo and rHDL post measurements, $^{*}P < 0.05$ from premeasurement within each infusion as measured by one-way ANOVA, n = 13. DAG, diacylglycerol; rHDL, reconstituted HDL; LPC, lysophosphatidylcholine; PC, phosphatidylcholine.

for placebo and rHDL, respectively); and NEFA (833 ± 49 and 1218 ± 115 μM for placebo and rHDL, respectively) following 4 h of rHDL infusion (Fig. 4), consistent with the composition of the rHDL infusate.

PC constituted 91% of all measured lipids in the rHDL infusate (**Table 2**), of which the major species (>95%) were 34:3, 34:2, 36:5, 36:4, 36:3, and 36:2, (**Table 3**). The 1.22 ± 0.09 mmol/l (173 ± 6.09%) increase in total PC after the rHDL infusion (Fig. 4) could be attributed to the 34:3, 34:2, 36:3, and 36:2 species; however, the expected increase in PC 36:4 was not observed (Table 3). Total plasma LPC increased (522 ± 32 μmol/l; 349 ± 19.6%; Fig. 4) after rHDL, and this approximated the observed increase seen in NEFA after rHDL (421 ± 75.8 μmol/l; 160 ± 10.1%; Fig. 4). LPC species containing 16:0, 18:0, 18:1, and 18:2 fatty acid chains accounted for ~500 μmol/l of the increase in total circulating LPC (**Table 4**), and the same four NEFA species account for ~350 μmol/l of the observed increase in total NEFA (**Table 5**).

DISCUSSION

The main finding from the present study was that an acute (4 hr) infusion of rHDL inhibited fasting-induced adipose tissue lipolysis and fat oxidation in humans with

TABLE 2. Lipidomic analysis of rHDL infusate

Lipid Species	Concentration (mmol/l) in rHDL Preparation	Percentage of All Lipids in rHDL Preparation
PC	34.7	91.4
LPC	2.34	6.2
COH-CE	0.002	0.02
DAG	0.35	0.92

COH-CE, cholesterol and cholesterol ester; DAG, diacylglycerol; rHDL, reconstituted HDL; LPC, lysophosphatidylcholine; PC, phosphatidylcholine.

type 2 diabetes. Inhibition of lipolysis possibly results from the dual effects of HDL-mediated insulin release (7) as well as activation of AMPK in adipose tissue. While insulin and AMPK act synergistically to reduce lipolysis in adipose tissue, it was not possible to determine which mechanism predominated after rHDL infusion. However, studies in adipose cell culture suggest for the first time that HDL increases AMPK signaling and inhibits lipolysis. The observed reduction in whole body fat oxidation after rHDL indicates a preponderance of an insulin effect over AMPK activation in skeletal muscle. Secondarily, this study also suggests that plasma enzyme-mediated turnover of PC moieties in the rHDL infusate leads to significant elevations in plasma NEFA.

Consistent with prolonged fasting, we observed a reduced plasma insulin level, together with increased rates of palmitate release (R_a), uptake (R_d), and oxidation in the placebo trial, indicative of increased adipose lipolysis and utilization of fatty acids as a substrate for ATP production. rHDL prevented this fasting-induced increase in lipolysis observed during the placebo infusion, as demonstrated by stable palmitate R_a, R_d, and oxidation rate. Because these data suggested that rHDL was potentially inhibiting fasting-induced lipolysis, two well-described plasma markers of lipolysis (42), NEFA and glycerol, were measured. Consistent with palmitate tracer data, the increases in plasma glycerol concentration observed during the placebo trial were abolished during the rHDL infusion. In this context, rHDL would also be expected to blunt fasting-induced elevations in NEFA; however, this was not observed, and in fact, NEFA was significantly increased beyond the placebo level by rHDL. Adipocyte cell culture studies were conducted to resolve this disparity and establish the direct effects of HDL on adipocyte lipolysis.

On the basis of previous data from our group and others, we hypothesized that HDL-mediated AMPK activation in adipocytes would inhibit lipolysis. This was confirmed with the demonstration that HDL both increased the phosphorylation of the AMPK target ACC and reduced NEFA and glycerol release from adipocytes. This inhibition was of a similar magnitude to that observed with AICAR and phenformin, two well-known activators of AMPK. While AMPK phosphorylation was not significantly increased by HDL, this finding is consistent with the previously well-described phenomenon that AMPK is generally transiently activated, with consequent sustained stimulation of downstream targets, such as ACC (7, 43, 44). The finding that AMPK inhibits lipolysis in adipocytes has been demonstrated in a number of previous studies in vitro (17, 45) and in vivo in rats (15). A recent study also demonstrated that systemic administration of AICAR in humans reduced whole body lipolysis in a manner similar to that observed in the current clinical trial (16). Given the similarities in the data from the study by Boon et al. and our current data, we believe this provides support in favor of rHDL acting through an AMPK-related mechanism in adipose tissue. To our knowledge, this is the first report to demonstrate that HDL can activate AMPK in adipocytes and inhibit

TABLE 3. PC species in the rHDL infusate and plasma PC species after 4 h infusion of rHDL or saline placebo

PC Species	Concentration (μmol/l) in rHDL Preparation	Percentage of Each PC Subspecies in rHDL Preparation	Concentration (μmol/l) Placebo	Concentration (μmol/l) rHDL	Change (μmol/l)	rHDL Percentage of Placebo
PC 30:2	5.56	0.02	4.75 ± 0.25	5.65 ± 0.33	0.9 ± 0.27	120 ± 6.1
PC 32:0[a]	199	0.58	13.4 ± 0.52	29.9 ± 1.38	16.5 ± 1.14	224 ± 8.9
PC 32:1[a]	34.5	0.1	29.1 ± 2.67	19.1 ± 1.42	−10.0 ± 1.9	68.7 ± 4.8
PC 32:2	122	0.35	7.55 ± 0.55	16.1 ± 0.8	8.53 ± 0.61	219 ± 11.1
PC 34:0[a]	313.0	0.90	32.6 ± 2.13	51.2 ± 2.5	18.6 ± 2.02	161 ± 8.3
PC 34:1[a]	401.9	1.2	49.0 ± 3.30	73.5 ± 3.21	24.5 ± 3.45	156 ± 9.7
PC 34:2[a,b]	7516	21.6	422 ± 28.0	965 ± 40.7	542 ± 42	237 ± 15.7
PC 34:3[a,b]	845	2.4	22.1 ± 1.77	117 ± 5.8	96.5 ± 5.45	567 ± 47.2
PC 36:2[a,b]	5863	16.9	248 ± 18.1	666 ± 29.8	418 ± 27.7	284 ± 22.5
PC 36:3[a,b]	4917	14.2	180 ± 11.0	567 ± 23.5	387 ± 22.5	326 ± 21.1
PC 36:4[a]	10745	31.0	162 ± 12.7	98.6 ± 5.73	−63.0 ± 12.1	64.5 ± 5.2
PC 36:5[a]	3500	10.1	35.2 ± 4.3	17.8 ± 3.36	−16.1 ± 4.7	52.8 ± 10.6
PC 38:4	60.2	0.17	120 ± 9.12	102 ± 7.24	−18.2 ± 7.9	87.2 ± 5.8
PC 38:5[a]	6.1	0.02	69.2 ± 5.86	50.5 ± 4.22	−18.7 ± 6.54	77.2 ± 7.6
PC 38:6[a]	0.74	<0.01	70.8 ± 6.5	45.8 ± 4.41	−25 ± 5.64	67.5 ± 5.3
PC 40:5	0.03	<0.01	15.5 ± 1.25	14.7 ± 0.8	−0.8 ± 1.16	100 ± 7.5
PC 40:6[a]	ND	ND	25.3 ± 2.53	21.5 ± 2.2	−3.69 ± 2.23	89.9 ± 7.3
PC 40:7[a]	ND	ND	4.75 ± 0.52	3.03 ± 0.29	−1.72 ± 0.43	68.1 ± 5.5
PC 44:12	ND	ND	1.46 ± 0.11	1.43 ± 0.15	−0.03 ± 0.12	98.4 ± 7.4

rHDL, reconstituted HDL; PC, phosphatidylcholine; ND, not detectable.
[a] 4 h values that were significantly different between rHDL and placebo infusions ($P < 0.05$).
[b] Species that made the majority contribution to the increase in plasma concentration seen after rHDL infusion, as indicated in "Results."

TABLE 4. Plasma LPC species after 4 h infusion of rHDL or saline placebo

LPC Species	Concentration (μmol/l) Placebo	Concentration (μmol/l) rHDL	Change (μmol/l)	rHDL Percentage of Placebo
LPC 14:0[a]	1.36 ± 0.14	2.49 ± 0.28	1.13 ± 0.25	197 ± 22.5
LPC 15:0[a]	1.5 ± 0.18	2.05 ± 0.15	0.55 ± 0.14	151 ± 15.3
LPC 16:0[a,b]	113 ± 9.87	200 ± 11.3	87.2 ± 9.01	185 ± 14
LPC 16:1[a]	3.83 ± 0.53	5.97 ± 0.52	2.14 ± 0.76	180 ± 17.8
LPC 18:0[a,b]	30.4 ± 3.21	49.3 ± 3.32	19.0 ± 2.55	176 ± 17.7
LPC 18:1[a,b]	26.1 ± 2.48	117 ± 6.93	90.9 ± 6.14	478 ± 39.6
LPC 18:2[a,b]	33.9 ± 3.68	342 ± 16.0	308 ± 14.7	1128 ± 116
LPC 20:0[a]	0.117 ± 0.01	0.670 ± 0.06	0.55 ± 0.05	645 ± 61.9
LPC 20:1[a]	0.27 ± 0.03	1.02 ± 0.08	0.75 ± 0.07	397 ± 34.7
LPC 20:2[a]	0.17 ± 0.02	0.32 ± 0.03	0.14 ± 0.02	193 ± 18.2
LPC 20:4[a]	8.6 ± 0.79	15.27 ± 1.13	6.67 ± 0.72	181 ± 10.2
LPC 22:6[a]	2.86 ± 0.27	4.78 ± 0.59	1.92 ± 0.43	169 ± 13.4

rHDL, reconstituted HDL; LPC, lysophosphatidylcholine.
[a]4 h values that were significantly different between rHDL and placebo infusions ($P < 0.05$).
[b]Species that made the majority contribution to the increase in plasma concentration seen after rHDL infusion, as indicated in "Results."

lipolysis. This direct effect would be reinforced by the additional actions of HDL on pancreatic insulin secretion (7).

The inconsistency between HDL-mediated inhibition of lipolysis in cell culture and the elevated NEFA after rHDL in the clinical trial implicated the rHDL infusate as the source of NEFA elevation. The infusate was prepared, as described previously (7), by combining human apoAI with soybean PC. This preparation undergoes rapid remodeling upon infusion (28), and we hypothesized that modifications to the infused PC would result in liberation of NEFA. LPC species compose only a small fraction of total phospholipids in the rHDL preparation, yet plasma concentrations of LPC were shown to rise substantially in conjunction with similar NEFA subspecies. Conversely, considering the high levels of PC species such as 34:6 in the rHDL infusate, these species did not rise in plasma to the levels that would have been expected after rHDL infusion. These data strongly imply that the actions of the phospholipase A2 super family on infused PC overrides the demonstrated inhibitory effect of HDL on lipolysis in vitro to elevate NEFA during rHDL infusion. NEFA would be derived from PC via the action of enzymes in the phospholipase A2 group, resulting in production of various NEFA and LPC species (46). While this analysis provides a snapshot for the circulating concentrations of various lipids, we acknowledge that it cannot characterize the complexity of lipid interactions occurring during the 4 h infusion period. These data strongly imply that the increased circulating NEFA observed after rHDL infusion is due to remodeling of the rHDL infusate and not due to direct signaling events elicited by rHDL. This finding thus allows us to more confidently interpret the accompanying data demonstrating that rHDL is indeed inhibiting lipolysis.

Taken together, the current data indicate that an acute infusion of rHDL significantly alters fasting-induced fatty acid turnover and oxidation. This is likely driven, at least in part, by inhibition of adipose tissue lipolysis as a result of activation of AMPK and HDL-induced increases in insulin secretion. These observations are consistent with the

TABLE 5. Plasma NEFA species after 4 h infusion of rHDL or saline placebo

NEFA Species	Concentration (μmol/l) Placebo	Concentration (μmol/l) rHDL	Change (μmol/l)	rHDL Percentage of Placebo
14:0[a]	11.8 ± 1.22	18.1 ± 2.09	6.3 ± 1.51	158 ± 15.7
15:0[a]	9.06 ± 1.12	13.68 ± 1.69	4.62 ± 1.77	182 ± 29.9
16:0[a,b]	313 ± 21.2	421 ± 36.8	108 ± 24.3	134 ± 7.18
16:1[a]	52.5 ± 5.59	68.3 ± 8.10	15.8 ± 5.48	131 ± 9.54
17:0[a]	7.72 ± 0.36	9.63 ± 0.76	1.90 ± 0.60	124 ± 7.81
18:0[a]	60.1 ± 2.93	83.2 ± 4.71	23.1 ± 3.91	140 ± 6.82
18:1[a,b]	292 ± 16.0	349 ± 26.9	56.2 ± 19.7	119 ± 6.52
18:2[a,b]	112 ± 8.50	284 ± 46.6	171 ± 39.7	240 ± 23.3
18:3[a]	22.2 ± 2.11	79.38 ± 16.3	57.2 ± 15.0	345 ± 47.8
19:0[a]	1.20 ± 0.17	1.91 ± 0.15	0.71 ± 0.21	203 ± 30.6
20:3	6.67 ± 0.88	8.10 ± 1.00	1.43 ± 0.36	124 ± 6.7
20:4	15.0 ± 1.69	16.8 ± 1.93	1.78 ± 0.66	112 ± 5.16
20:5	3.33 ± 0.48	4.08 ± 0.54	0.74 ± 0.50	165 ± 41.9
22:5[a]	4.86 ± 0.37	6.31 ± 0.57	1.45 ± 0.47	133 ± 10.9
22:6	9.98 ± 0.95	13.2 ± 1.88	3.18 ± 1.22	129 ± 9.79

rHDL, reconstituted HDL.
[a]4 h values that were significantly different between rHDL and placebo infusions ($P < 0.05$).
[b]Species that made the majority contribution to the increase in plasma concentration seen after rHDL infusion, as indicated in "Results."

hypothesis that low plasma levels of HDL observed in patients with type 2 diabetes may contribute to increased circulating NEFA and the pathophysiology of type 2 diabetes. However, while this study highlights the capacity for rHDL to modulate fatty acid metabolism in adipocytes, the phospholipid component of the rHDL infusate did directly increase circulating NEFA. Our findings suggest that HDL elevating agents not associated with PC infusion, such as CETP inhibitors or apoAI transcriptional upregulators, may also inhibit adipocyte lipolysis. Detailed investigation of fatty acid metabolism is therefore warranted in future investigations of chronic HDL-elevating agents.

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