Effect of R(+)α-Lipoic Acid on Pyruvate Metabolism and Fatty Acid Oxidation in Rat Hepatocytes

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R-(+)-α-lipoic acid (R-LA) is the naturally occuring enantiomer of LA. It is a strong antioxidant and cofactor of key metabolic enzyme complexes catalyzing the decarboxylation of α-keto acids. Racemic LA (rac-LA) has shown promise in treating diabetic polyneuropathy, and some studies suggest that it improves glucose homeostasis in patients with type 2 diabetes. We examined the effects of R-LA on pyruvate metabolism and free fatty acid (FFA) oxidation in primary cultured hepatocytes isolated from 24-hour fasted rats. After overnight culture in serum-free medium, cells were pre-exposed to R-LA for 3 hours before assays. R-LA (25 to 200 μmol/L) significantly increased pyruvate oxidation ($\sim$2-fold at the highest dose tested) measured as $^{14}CO_2$ production from [1-^{14}C]pyruvate by the cells over 1 hour post-treatment. These effects correlated with proportional, significant increases in the activation state of the pyruvate dehydrogenase (PDH) complex. R-LA treatment inhibited glucose production from pyruvate by approximately 50% at 50 μmol/L R-LA and approximately 90% at 200 μmol/L. Palmitate oxidation was measured in hepatocytes cultured in the presence of albumin and physiological (0.1 mmol/L) or high (1.5 mmol/L) concentrations of FFA. The latter markedly enhanced FFA oxidation. R-LA treatment significantly inhibited FFA oxidation in both media, but was more effective in high FFA, where it reduced FFA oxidation by 48% to 82% at 25 to 200 μmol/L, respectively. Identical doses of R-LA did not affect FFA oxidation by L6 myotubes (a cell culture model for skeletal muscle) in either high or low FFA medium, but enhanced pyruvate oxidation. In conclusion, 3-hour exposure of primary cultured rat hepatocytes to R-LA at therapeutically relevant concentrations increased pyruvate oxidation, apparently by activation of the PDH complex, and decreased gluconeogenesis and FFA oxidation. These features may prove useful in the control of type 2 diabetes.

R-(+)-α-lipoic acid (thioctic acid; R-LA) is an endogenous cofactor of key metabolic enzyme complexes catalyzing the decarboxylation of α-keto acids, ie, pyruvate, α-ketoglutarate, and branched-chain α-ketoacid dehydrogenase complexes. Due to its antioxidant properties,[1] LA is thought to be potentially useful in treating several brain and nerve disorders, including diabetic polyneuropathy. In short-term studies, the readily available racemic mixture of LA (rac-LA $>$ 600 mg/d) showed promise in treating polyneuropathy by producing significant reductions in neuropathic symptoms in diabetic patients.[2,3] In a rat model of diabetic polyneuropathy, treatment with rac-LA increased the conduction velocity of sensory nerves and improved several metabolic parameters in peripheral nerves of diabetic rats.[4] In rats with streptozotocin-induced diabetes, dietary supplementation with rac-LA was effective in preventing early glomerular injury.[5] In cultured human aortic endothelial cells, addition of R-LA to the culture medium prevented tumor necrosis factor-alpha (TNF-α)–induced nuclear factor κB (NF-κB) activation and adhesion molecule expression.[6] These and other effects[1] have been attributed to the antioxidant properties of LA. LA may also affect glucose disposal and metabolism. In fact, pilot studies using rac-LA suggest that treatment of type 2 diabetic patients with LA may increase insulin-stimulated glucose disposal,[7] enhance glucose effectiveness, and decrease serum lactate and pyruvate levels in response to a glucose load.[8]

It has been proposed that the metabolic effects of LA reflect in part activation of pyruvate dehydrogenase (PDH). In diabetic patients, enhanced PDH activity would increase glucose metabolism and decrease glucose production and free fatty acid (FFA) oxidation. It has been difficult for investigators to establish with confidence that LA positively influences metabolism in diabetic patients. This may be explained by several factors, including the limited size and length of the studies, and possibly their utilization of rac-LA consisting of R- and S-LA in approximately equal proportions. If the metabolic effects of LA reflect in part enhancement of PDH activity, the use of the purified R-enantiomer may be beneficial, as the PDH complex may have a catalytic preference for the R-enantiomer, and even opposing effects between the 2 enantiomers have been suggested.[9-13]

In addition to more extensive clinical studies using R-LA, further laboratory studies designed to investigate its metabolic effects and mechanism of action are needed. To date, the effect of LA on pyruvate metabolism in hepatocytes has been studied only using very high concentrations (1 mmol/L) of rac-LA for short exposure times in suspension culture,[14] and effects on PDH have mainly been examined using the purified enzyme complex.[10,13] It is of interest to determine the effects of the purified R-enantiomer on glucose and pyruvate metabolism in differentiated hepatocyte monolayer cultures. In the following study we determine the effect of R-LA (Dexlipotam; Viatris

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AG, Frankfurt, Germany) on the decarboxylation of pyruvate, the proportion of the PDH complex in the active form, the production of glucose from pyruvate, and FFA oxidation in rat hepatocytes.

MATERIALS AND METHODS

Materials

Perchloric acid and sulfuric acid were obtained from Fisher Biotech (Fairlawn, NJ). Collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Williams' minimal essential medium (WME), Dulbecco's phosphate-buffered saline (DPBS), fetal bovine serum (FBS), Hank's balanced salt solution, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). Dowex 50 × 8 and Duolite A7 resins were obtained from Supelco (Bellefonte, PA). Male Long Evans Hooded rats were obtained from Charles Rivers Laboratories (Wilmington, MA). R(+)α-lipoic acid-Tris salt (Dexlipotam, R-LA) was a gift of Viatris AG. S-LA and rac-LA were also gifts of Viatris AG and were tested in some experiments as Tris salts. Radiolabeled substrates, [1-^{14}C]pyruvic acid (specific activity, 10 to 30 mCi/mmol) and [9, 10 (n)-^{3}H]palmitic acid (40 to 60 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ). Except where noted, all other chemicals were obtained from Sigma Chemical Co (St Louis, MO) and were of the highest purity available.

Hepatocyte Isolation and Culture

Hepatocytes were isolated from livers of male Long Evans Hooded rats (150 to 250 g) by collagenase perfusion as previously described.[15] Rats were fasted for 24 hours before experiments, unless otherwise indicated in the text. They were anesthetized with pentobarbital (intraperitoneally, 50 mg/kg) prior to liver perfusion. Hepatocytes were separated from other cell types by centrifugation at 50 × *g* for 2 minutes at 4°C. Viability of the hepatocytes was determined by trypan blue exclusion; only preparations with viability greater than 80% were used. The cells were plated on collagen-coated plates at approximately 8×10^4 cells/cm^2 in WME containing 10% FBS, 2 mmol/L L-glutamine, 34 ng/mL insulin, and 0.1 mg/mL gentamicin sulfate. After a 2-hour attachment period, media and unattached cells were removed and media was replaced with serum-free WME containing 2 mmol/L L-glutamine, 300 μmol/L bovine serum albumin (BSA; Intergen, Purchase, NY), 0.1 mg/mL gentamicin, and 5 mmol/L sodium pyruvate with or without the addition of 0.05 to 0.75 mmol/L oleic and palmitic acid (oleate and palmitate were added in equal concentrations for a total FFA concentration of 0.1 to 1.5 mmol/L). FFAs were dissolved with heating in a small volume (2 mL) of DPBS and added directly, with stirring, to 100 mL pre-equilibrated WME containing 300 μmol/L BSA. An equal volume of DPBS was added to control media containing no FFAs.

L6 Cell Culture

Cryopreserved L6 myocytes/myoblasts were a kind gift of Dr Amira Klip. The cells were passaged according to Mitsumoto and Klip[16] in minimal essential medium (MEM)-alpha growth medium (Gibco) containing 100 U/mL penicillin, 100 μg/mL streptomycin, 250 μg amphotericin, and 10% FBS. To obtain differentiated myotube cultures, cells were seeded at 20,000 cells/ mL into MEM-alpha medium containing 2% FBS and maintained in culture for at least 1 week postconfluency before experimentation.

Hepatocyte Treatments

Hepatocytes remained in serum-free WME containing 2 mmol/L L-glutamine, 300 μmol/L BSA, 0.1 mg/mL gentamicin, 5 mmol/L sodium pyruvate in the presence or absence of FFA overnight. Treatment was initiated the following morning, approximately 22 hours after cell plating. R-LA (0 to 200 μmol/L) was added in a small amount of argonated control medium (as described above, without FFA). Drug concentrations and duration of treatment were determined to be noncytotoxic through measurement of lactic acid dehydrogenase (LDH) release into the medium (data not shown). Following treatment (3 hours in the experiments shown here) parameters of metabolic activity were measured in drug-free media. However, in other experiments (not shown) we established that the presence of R-LA during the assays did not modify the results.

Measurement of $^{14}CO_2$ Production From [1-^{14}C]Pyruvate

$^{14}CO_2$ production from [1-^{14}C]pyruvate was measured in hepatocytes grown in 6-well tissue culture plates using modifications of a previously described assay.[17] Following treatment for 3 hours, cells were placed into identical media, but without R-LA, and supplemented with 0.5 μCi [1-^{14}C]pyruvate/2 mL/well. Blank wells containing each permutation of the cell media but no cells were included. After 1-hour incubation (37°C), media from each well was collected and placed into rubber-stoppered vials with center wells. Hyamine hydroxide (200 μL of 1-mol/L solution in methanol; Research Products International, Mt Prospect, IL) was injected into each center well, and then 200 μL of 10% perchloric acid (PCA) was injected into the media. The vials were incubated at 37°C for 90 minutes with agitation. The released $^{14}CO_2$ was absorbed in hyamine and counted in a scintillation counter. Cells from each well were scraped into 1 mL of DPBS and centrifuged at 2,500 rpm. Cell pellets were resuspended in lysis buffer containing 20 mmol/L Tris, 25 mmol/L β-glycerophosphate, 137 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L pyrophosphate, 1 mmol/L vanadate, 1% Triton X-100, 10% glycerol, and 1 mmol/L dithiothreitol (DTT), and centrifuged at 14,000 rpm for 15 minutes. The supernatant cell extract was assayed for protein using a Coomassie protein assay (Pierce, Rockford, IL) and BSA standards.

For measurement of pyruvate oxidation by L6 myotubes, cells maintained in MEM-α medium containing 2% FBS, were serum deprived for 2 hours in Dulbecco's MEM (DMEM) containing 2 mmol/L L-glutamine, 5 mmol/L glucose, and 1 mmol/L pyruvate. Cells were then exposed for 3 hours to the same medium with or without R-LA at the concentrations indicated, and then for 1 hour to DMEM containing 2 mmol/L L-glutamine, 1 mmol/L pyruvate, 1 mmol/L glucose, and 0.5 μCi of [1-^{14}C] pyruvate per well, without R-LA. The assay was then performed as described above for hepatocytes.

In all experiments, $^{14}CO_2$ production from the media incubated without cells was measured, and subtracted as blank from $^{14}CO_2$ production by the cells. R-LA did not affect blank $^{14}CO_2$ production. Blanks varied between 10% and 40% of counts recovered from control cells, incubated without R-LA, in different experiments.

Measurement of Percent Activation of the PDH Complex

The assay was modified from previous publications.[18-20] Hepatocytes plated in 150-mm tissue culture plates were treated for 3 hours without or with R-LA (25 to 100 μmol/L). The plates were rinsed in DPBS and the cells were flash frozen on the plate by dipping them in liquid nitrogen. The cells were immediately scraped into ice-cold buffer containing 50 mmol/L HEPES/KOH (pH 7.5) with 5 mmol/L DTT, 3 mmol/L EDTA, 2% Triton X-100, and 10 μg/mL aprotinin and leupeptin. Cells from half of the plates in each treatment group were scraped into identical buffer supplemented with 0.1 mol/L KF to prevent activation of the PDH complex by phosphatase action. To fully activate the PDH complex by activating PDH-phosphatase, the sample lacking KF was made 10 mmol/L in $MgCl_2$ and 0.1 mmol/L in $CaCl_2$. Samples were mixed and then immediately frozen by immersion in liquid nitrogen, thawed, mixed again, and then homogenized on ice for 10 seconds with a Polytron homogenizer (setting 3). Both tubes were

incubated for 30 minutes at 37°C. A small aliquot of each sample was taken for protein measurement using a Coomassie protein assay.

The assay mixure for measurement of PDH activity contained the following: 30 mmol/L KH_2PO_4, 2 mmol/L $MgCl_2$, 0.4 mmol/L thiamin pyrophosphate, 0.4 mmol/L coenzyme A (CoA), 1 mmol/L DTT, 1 mmol/L nicotinamide adenine dinucleotide (NAD), 0.1% Triton X-100, and 0.04 mg/mL lipoamide dehydrogenase. Following incubation, 1.5 mg protein from each sample was added to 2 mL of the above reaction mixture. Vials (including blanks containing no hepatocyte protein) were loosely sealed with a rubber stopper, and the samples pre-equilibrated for 10 minutes in a shaker bath at 30°C. After pre-incubation the reaction was initiated by adding 5 mmol/L unlabeled pyruvate and 0.5 μCi of [1-^{14}C]pyruvate, and each flask was sealed tightly with a rubber stopper fitted with a suspended center well. After 15 minutes, 0.2 mL of hyamine hydroxide was injected into each center well, and the reaction was stopped by injecting 1 mL 5-mol/L H_2SO_4 into the media. $^{14}CO_2$ released from the medium was collected for 1 hour. The center wells were then removed, placed into 10 mL Scintisafe 30% scintillation fluid (Fisher Scientific, Suwanee, GA), vortexed, and counted. All extracts in each treatment group were measured in duplicate. Radioactivity recovered from blanks (without cell extract) was subtracted as background from all sample counts. The activation state of the PDH complex was expressed as the ratio of (net dpm in KF treated/net dpm in $CaCl_2$ + $MgCl_2$ activated samples) × 100.

Measurement of ^{14}C-Glucose Production From [1-^{14}C]Pyruvate

For studies measuring gluconeogenesis from pyruvate, hepatocytes were plated in 6-well tissue culture plates. [1-^{14}C]pyruvate incorporation into glucose was measured using a modification of an assay described by Exton and Park.[21] After treating the cells for 3 hours with or without R-LA, medium was changed to DMEM containing 1 mmol/L glucose + 1.5 mmol/L pyruvate + 0.5 μCi [1-^{14}C]pyruvate ± R-LA where appropriate. Media was collected from wells 2 hours later and either analyzed immediately or frozen at −80°C for no more than 1 week. Cells from each well were scraped into 1 mL of DPBS and centrifuged at 2,500 rpm. Cell pellets were resuspended in lysis buffer, centrifuged, and the supernatants (whole cell extracts) used to determine protein concentrations.

Media were deproteinized by adding 15 μL of 10% PCA/500 μL of sample. PCA was extracted from the supernatants with 1 mL of a 1:3 mixture of tri-n-octylamine: 1,1,2-trichlorotrifluoroethane and centrifuged at 14,000 rpm; 350 μL of the upper aqueous layer was placed into an Eppendorf tube containing 53.3 mg Dowex 50 × 8 resin and 106.6 mg Duolite A7 resin. Samples were mixed for 1.5 hours and then centrifuged at 2,500 rpm for 3 minutes; 150 μL of the supernatant was placed into Scintisafe (30%) scintillation fluid and radioactive counts were assessed. In several experiments, glucose in the supernatant was quantified by the glucose oxidase method using a glucose analyzer (YSI 2300, Yellow Spring Instruments, Yellow Springs, OH).

Measurement of ^3H_2O Release From ^{3}H-Palmitic Acid

To quantify FFA oxidation, a modified assay of Sreenan et al was used.[22] Hepatocytes were plated in 6-well tissue culture plates. After 3 hours treatment with or without R-LA, media in each well were replaced with media containing 0.5 μCi of ^{3}H-palmitic acid. Two hours later, 500 μL of media was collected from each well. Media were either analyzed immediately or frozen at −80°C for no more than 1 week. Cells from each well were scraped into 1 mL of DPBS and the cell pellets were lysed and analyzed for protein as described above.

Proteins were precipitated from each sample of medium with PCA, as described above. The supernatants were extracted with methanol: chloroform (1:3), vortexed, centrifuged at 14,000 rpm for 3 minutes, and 500 μL of the upper aqueous layer was placed into 10 mL of Scintisafe (30%) scintillation fluid and counted for radioactivity.

For measurement of FFA oxidation by L6 myocytes, the cells were cultured overnight in differentiation medium that had been modified to contain 5 mmol/L pyruvate, 300 μmol/L BSA (for FFA binding), and either 0.1 mmol/L FFA ("physiological" concentration) or 1.0 mmol/L FFA ("high" FFA). The FFA were an equal mixture of oleate and palmitate as in hepatocyte experiments. The following morning media were changed to serum-free MEM-α of the same composition as above without or with R-LA (25, 50, or 100 μmol/L). After 3 hours, these media were replaced with identical media supplemented with [^{3}H]palmitic acid (2 μCi/well). After 2 hrs incubation, media and cells were harvested and analyzed as in hepatocyte experiments.

Statistical Analyses

Means ± SEM are shown. The significance of differences between means was evaluated by 2-tailed, unpaired Student's *t* test and by 1-way analysis of variance (ANOVA) (Microsoft Excel 2000; Redmond, WA). $P < .05$ was considered significant.

RESULTS

The effects of 3 hours treatment with R-LA following a 20-hour preincubation in serum-free WME containing 300 μmol/L BSA, 5 mmol/L pyruvate ± 1.5 mmol/L FFA (0.75 mmol/L palmitate and oleate) on the decarboxylation of pyruvate are illustrated in Fig 1. Data are expressed as nanomoles of $^{14}CO_2$ produced per milligram of cellular protein assuming that the specific activities of pyruvate in the medium and in the mitochondria are at equilibrium. One-way ANOVA indicated that R-LA addition significantly enhanced pyruvate decarboxylation in control media ($P < .01$) but not in FFA-supplemented media ($P = .134$). When R-LA–treated samples were compared to their respective untreated controls in the presence of 1.5 mmol/L FFA, R-LA significantly increased pyruvate decarboxylation at 25 μmol/L ($P < .05$) and 100 μmol/L ($P < .05$) only, while in control medium, R-LA stimulated $^{14}CO_2$ production in a dose-dependent manner with significant enhancement at 25 ($P < .05$), 50 ($P < .01$), 100 ($P < .01$), and 200 ($P < .001$) μmol/L R-LA. Maximal stimulation (following incubation with 200 μmol/L R-LA) was approximately 2-fold compared to control.

To assess whether this increase in pyruvate decarboxylation reflects activation of the PDH complex, we measured PDH activity in extracts prepared in the presence of fluoride from control and R-LA–treated cells and compared this to the total potential PDH activity in cells treated in the same manner. By 1-way ANOVA, the percentage of activated PDH increased significantly with R-LA treatment ($P < .05$). The effect was significant (compared to untreated controls) in response to 50 μmol/L or 100 μmol/L R-LA ($P < .05$ by 2-tailed Student's *t* test) (Fig 2), and was of similar magnitude as the increase in pyruvate flux (Fig 1).

We next examined glucose production from pyruvate in the hepatocyte cultures. As expected, fasting the rat prior to hepatocyte isolation increased gluconeogenesis. Glucose production from pyruvate increased linearly with time between 1 and 3 hours incubation (Fig 3A). One-way ANOVA revealed that R-LA treatment significantly reduced the conversion of pyruvate into glucose by hepatocytes from fasted rats ($P < .0001$) (Fig 3B). The decrease in gluconeogenesis was dose-dependent

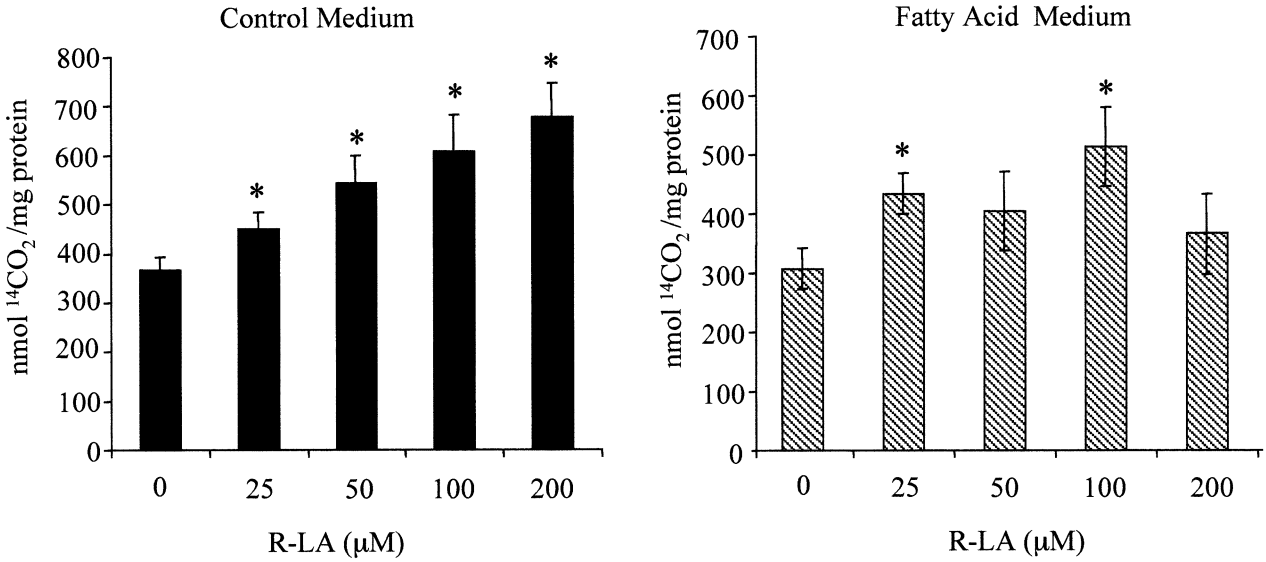

Fig 1. Effects of preincubation with R-LA in control or high fatty acid media on pyruvate decarboxylation. Hepatocytes isolated from 24-hour–fasted rats were preincubated for 20 hours in WME containing either 300 μmol/L BSA and 5 mmol/L pyruvate (control medium) or 300 μmol/L BSA, 5 mmol/L pyruvate, and 0.75 mmol/L each of oleate and palmitate (fatty acid medium). Cells were then incubated in the respective fresh medium (control or fatty acid) with the addition of 0 to 200 μmol/L R-LA for 3 hours and then placed into identical media (without R-LA) containing 0.5 μCi of [1-^{14}C]pyruvate per well. $^{14}CO_2$ was collected from the medium 1 hour later. Means ± SEM are shown; n = 8 to 9 per group, from three separate experiments. *$P < .05$ compared with appropriate control from each medium.

with significant reduction at 50 ($P < .05$), 100 ($P < .01$), and 200 ($P < .01$) μmol/L R-LA. Inhibition at the highest dose tested was approximately 90%. R-LA also decreased glucone-

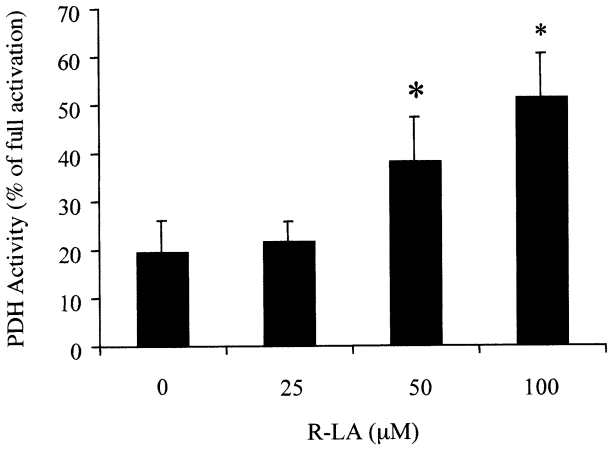

Fig 2. Effects of preincubation with R-LA on PDH complex activity. Hepatocytes isolated from 24-hour–fasted rats were preincubated for 20 hours in WME containing 300 μmol/L BSA and 5 mmol/L pyruvate (control medium). Cells were then incubated in fresh medium with the addition of 0 to 200 μmol/L R-LA for 3 hours. Cells were then flash frozen, harvested, and homogenized; half of each homogenate sample was treated with KF (to inhibit PDK-phosphatase), which was activated in the other aliquot by adding $CaCl_2$ and $MgCl_2$. $^{14}CO_2$ production from [1-^{14}C]pyruvate was measured in both aliquots from each treatment group. Data is expressed as the percentage of active PDH complex. Means ± SEM are shown; n = 4-5/treatment group from 4 to 5 separate experiments. *$P < .05$ compared to control from each medium.

ogenesis in hepatocytes from ad libitum–fed rats in a similar dose-dependent fashion (data not shown). The decrease in ^{14}C-glucose production from [1-^{14}C]pyruvate was corroborated by measurements of total glucose in the medium collected at the end of the experiments (Fig 3C). Data are expressed as the difference in glucose concentration (Δglucose) between media incubated with cells in the different treatment groups, and media incubated without cells ("blank" media), for 2 hours. Clearly, control hepatocytes produced much more glucose than they utilized from the medium. Glucose release into the medium decreased in cells pretreated with R-LA in a dose-dependent manner ($P < .05$) at all concentrations tested, similar to the data shown in Fig 3B. Cells pretreated with 100 or 200 μmol/L R-LA were in negative balance and removed glucose from the medium.

We also examined the effect of R-LA on FFA oxidation by hepatocyte cultures, in the presence of physiological (0.1 mmol/L) or high (1.5 mmol/L) concentrations of FFA in the media. ^3H_2O production from ^{3}H-palmitate was measured. High FFA increased palmitate oxidation ($P < 1 \times 10^{-7}$) (Fig 4). In both normal and high FFA media, R-LA treatment significantly reduced FFA oxidation (1-way ANOVA; 0.1 mmol/L FFA media, $P < .001$; 1.5 mmol/L FFA media, $P < 1 \times 10^{-11}$). In high FFA media, R-LA decreased palmitate oxidation significantly at all concentrations tested (25 μmol/L, $P < .001$; 50 to 200 μmol/L, $P < .0001$), while in normal FFA media, only 100 μmol/L ($P < .05$) and 200 μmol/L ($P < .01$) R-LA treatment reduced FFA oxidation significantly. In high FFA medium, treatment with 25 μmol/L R-LA reduced FFA oxidation by approximately 50%, and 200 μmol/L R-LA treatment caused approximately 85% inhibition. At the highest dose

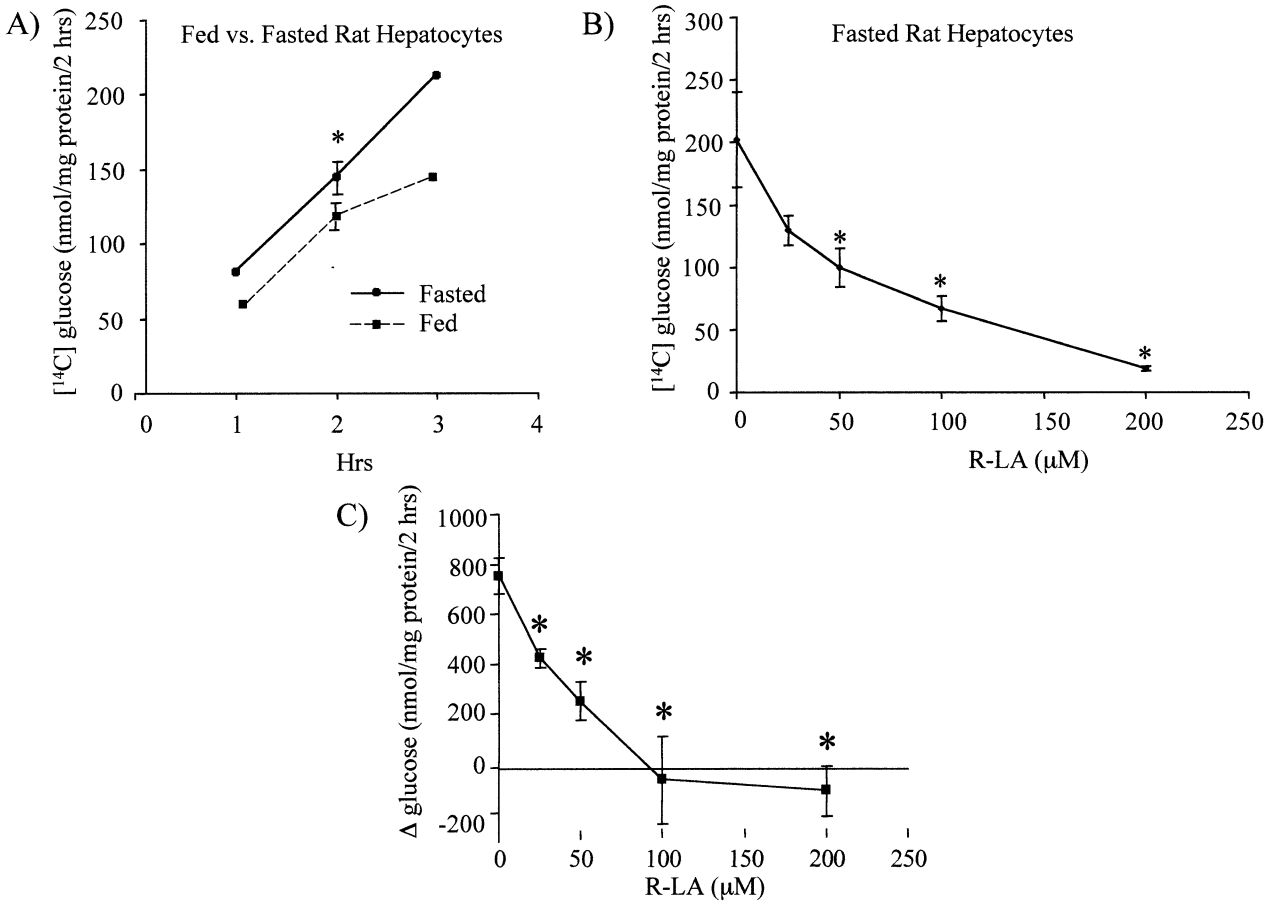

Fig 3. (A) Time course of glucose production from pyruvate in hepatocytes from fasted *v* nonfasted rats. Hepatocytes from nonfasted or 24-hour–fasted rats were preincubated for 20 hours in WME containing 300 μmol/L BSA, 5 mmol/L glucose, and 5 mmol/L pyruvate. Medium was refreshed, and then 3 hours later the medium was changed to DMEM containing 1 mmol/L glucose and 1.5 mmol/L [1-^{14}C]pyruvate (0.25 μCi/mL). Media was collected 1, 2, and 3 hours after ^{14}C-pyruvate addition and analyzed for ^{14}C-glucose content. Means are shown at all 3 time points; mean ± SEM is shown at the 2-hour time point n = 3; n = 2 for other time points. *P < .05 compared to hepatocytes from nonfasted ("fed") rats. (B) Effect of preincubation with R-LA on glucose production from pyruvate. Hepatocytes from 24-hour–fasted rats were preincubated for 20 hours as in (A) and then for 3 hours in identical media ± R-LA (25 to 200 μmol/L). Media was replaced with DMEM containing 1 mmol/L glucose and 1.5 mmol/L [1-^{14}C]pyruvate (0.25 μCi/mL). Two hours later media was collected and analyzed for ^{14}C-glucose content. Means ± SEM are shown; n = 8 for control, 50 and 100 μmol/L R-LA-treated groups, and n = 5 for 25 and 200 μmol/L R-LA treatments, from 3 to 4 separate experiments. *P < .05 compared to controls. (C) Effect of R-LA pretreatment on net glucose production/utilization by hepatocytes. Media collected from studies in (B) was analyzed for total glucose concentration by the glucose oxidase method. ΔGlucose was determined as the difference in glucose (nmol/2 mL) between media incubated with hepatocytes for 2 hours and media incubated without cells and normalized to cell protein (~0.15 mg/well). Positive values indicate net glucose production and negative values net glucose utilization. Means ± SEM are shown; n = 6 from 3 separate experiments. *P < .05 compared to untreated hepatocytes.

tested (200 μmol/L) R-LA inhibited FFA oxidation by approximately 50% in low-FFA–containing medium.

In contrast to our findings in hepatocytes, R-LA had no effect on palmitate oxidation in L6 myotubes (Fig 5A). Treatment of the L6 myotubes with 1.0 mmol/L FFA increased palmitate oxidation approximately 5-fold versus 0.1 mmol/L FFA. R-LA treatment did not alter palmitate oxidation at either physiological (0.1 mmol/L) or high (1.0 mmol/L) concentrations of FFA in the media.

Because the above findings suggested that R-LA may not be taken up by L6 myotubes, we studied the effect of R-LA treatment on pyruvate oxidation by these cells (Fig 5B). While 25 μmol/L R-LA was ineffective, 50 and 100 μmol/L R-LA increased $^{14}CO_2$ production from [1-^{14}C] pyruvate by 51% and 75%, respectively, as compared to control cells, incubated without R-LA ($P < .005$ and $< .001$, by 2-tailed Student's t test).

To assess whether stimulation of pyruvate oxidation was specific for the R-enantiomer of LA, in preliminary studies we compared $^{14}CO_2$ production by isolated hepatocytes incubated with equimolar rac-LA, S-LA, or R-LA (all as Tris-salts). The experimental conditions were as described in Fig 1. In a typical experiment (n = 3 per group), 100 μmol/L rac-LA stimulated $^{14}CO_2$ production by 73% ± 11%, S-LA by 65% ± 7.5%, and R-LA by 100% ± 14%. Corresponding values at 50-μmol/L drug concentration were 38% ± 7%, 68% ± 6%, and 79% ±

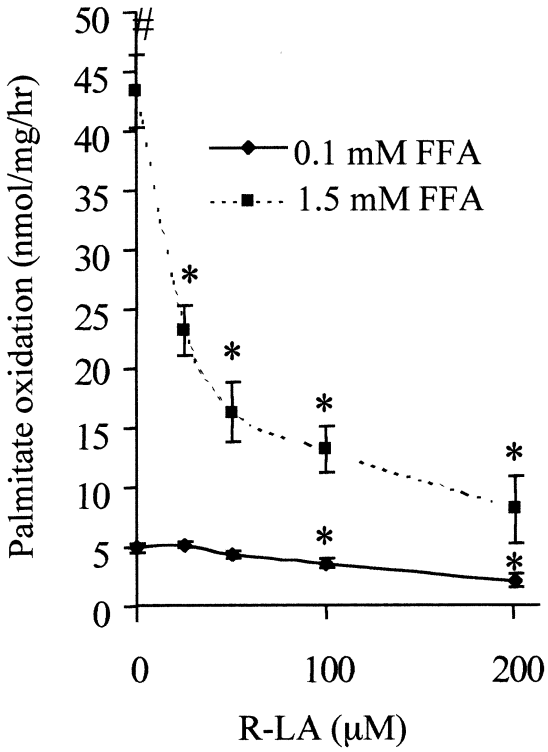

Fig 4. Effects of preincubation with R-LA in normal (0.1 mmol/L) or high (1.5 mmol/L) FFA-containing medium on FFA oxidation. Hepatocytes isolated from 24-hour–fasted rats were preincubated for 20 hours in WME containing 300 μmol/L BSA, 5 mmol/L glucose, 5 mmol/L pyruvate, and supplemented with either 0.05 mmol/L oleate and palmitate (0.1 mmol/L FFA medium) or with 0.75 mmol/L oleate and palmitate (1.5 mmol/L FFA medium) and then incubated for 3 hours in identical media ± R-LA (25 to 200 μmol/L). Media was then refreshed, 0.5 μCi of ^{3}H-palmitate was added per 2 mL well and the media collected 2 hours later for measurement of ^3H_2O. Means ± SEM are shown; n = 12 for control, 50 and 100 μmol/L R-LA–treated groups, and n = 6 for 25 and 200 μmol/L R-LA treatments, from 3 to 4 separate experiments. *P < .05 compared with appropriate control from each medium. #P < .05 compared to normal (0.1 mmol/L) FFA medium.

15%. While stimulation over control was significant for each preparation ($P < .02$), there was no statistically significant difference between the effects of the 3 compounds. Thus while we cannot rule out the possibility that R-LA may be slightly more effective than S-LA in this model, the major effect appears to be shared by R- and S-LA.

DISCUSSION

Despite several studies focused on the antioxidant properties of rac-LA, little is known of the metabolic consequences of exposure to exogenous R-LA and its potential to improve glycemic control in diabetic patients. Some studies suggested that treatment with rac-LA improves insulin sensitivity[7] and glucose effectiveness[8] and decreases serum lactate and pyruvate[8] in patients with type 2 diabetes. Studies in insulin-resistant obese Zucker rats reported enhanced insulin-stimulated glucose uptake, glycogen synthesis, and glucose oxidation by skeletal muscle, and decreases in circulating insulin and FFA levels after chronic treatment with R-LA.[11] In fasted nondiabetic and streptozotocin-induced diabetic rats, short-term intravenous administration of rac-LA caused hypoglycemia without

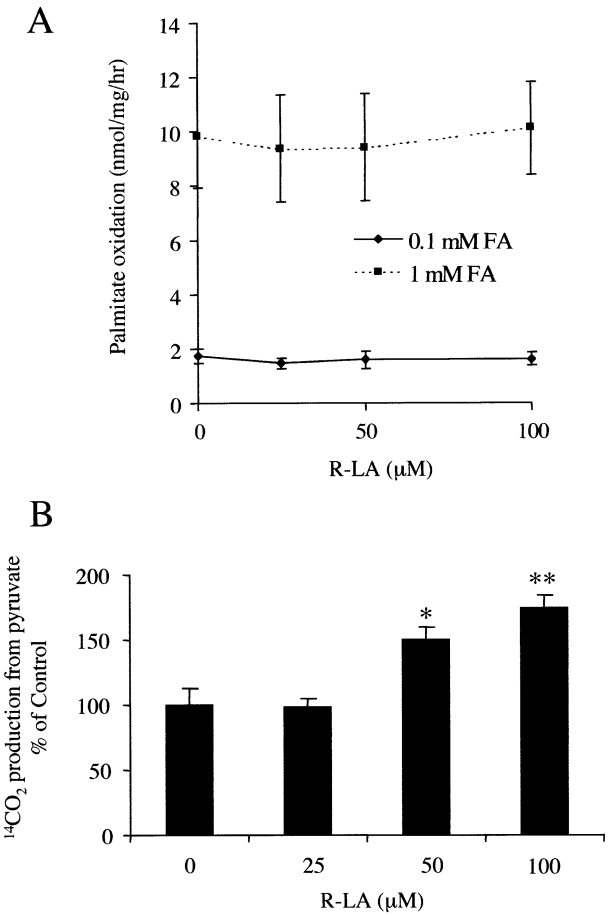

Fig 5. (A) Effects of preincubation with R-LA in normal (0.1 mmol/L) or high (1.0 mmol/L) FFA-containing media on FFA oxidation by L6 myotubes. Cells were incubated overnight in differentiation medium modified to contain 5 mmol/L glucose, 5 mmol/L pyruvate, 300 μmol/L BSA, and either 0.1 mmol/L FFA ("physiological" concentration) or 1.0 mmol/L FFA ("high" FFA). The following morning media were changed to serum-free MEM-α of the same composition without or with R-LA (25, 50, or 100 μmol/L). After 3 hours, the medium was replaced with media of identical composition (including R-LA where present) supplemented with [^{3}H]palmitate (2 μCi/2mL/well). Media was collected 2 hours later and analyzed for ^3H_2O. Means ± SEM are shown; n = 11 from 4 separate experiments. #P < .05 compared to normal (0.1 mmol/L) FFA medium. (B) Effect of preincubation with R-LA on pyruvate decarboxylation by L6 myotubes. Myotubes were kept overnight in MEM-α medium containing 2% FBS and then serum starved for 2 hours in DMEM containing 5 mmol/L glucose and 1 mmol/L pyruvate. They were then placed into identical media without or with R-LA at the concentrations indicated for 3 hours, and then into DMEM without R-LA, containing 1 mmol/L glucose, 1 mmol/L pyruvate, and 0.5 μCi of [1-^{14}C] pyruvate per well. $^{14}CO_2$ was collected from the media 1 hour later. Data (nmol $^{14}CO_2$ /mg protein/h) were normalized in each experiment to $^{14}CO_2$ production by control cells, not exposed to R-LA. Means ± SE are shown, n = 12 from 4 separate experiments. Mean $^{14}CO_2$ production by control cells was 149 ± 20 nmol/mg protein/h. *P < .005, **P < .001, compared to control.

a change in circulating insulin levels.[23] In cell culture studies, enhanced glucose uptake following R-LA treatment has been reported in L6 myotubes[24] and 3T3-L1 adipocytes.[25]

As the liver plays a central role in controlling glucose metabolism, we examined the metabolic effects of R-LA in a cell culture model of primary rat hepatocytes. The only other reported investigation of the metabolic effects of LA in hepatocytes involved hepatocyte suspension cultures treated with rac-LA.[14] The current study used confluent monolayer hepatocyte cultures maintained in differentiated condition (serum-free medium) and treatment with physiologically relevant concentrations of the purified R-enantiomer of LA. The maximal serum LA concentration observed in humans treated with R-LA (300 to 600 mg as a single daily oral dose for 1 to 3 weeks) has been approximately 25 to 50 μmol/L. Maximal serum concentrations were observed 30 to 60 minutes after drug ingestion (Dr Mathias Locher, Viatris AG, personal communication). This dose of R-LA is similar to the doses of rac-LA used in previous clinical studies where serum concentrations of LA were not determined.[7,8]

We used rats fasted for 24 hours prior to hepatocyte isolation to induce metabolic changes in the cells that would simulate those seen in the diabetic state. In liver, starvation, like diabetes, is known to induce a highly active isoform (PDK4) of pyruvate dehydrogenase kinase, the enzyme responsible for phosphorylating and thereby inactivating the PDH complex.[26,27] This inactivation is critical in diabetes, where it exacerbates the imbalance in glucose homeostasis by conserving glucose and gluconeogenic substrates. In some experiments, exposure of the hepatocytes to high concentrations of FFA was also used to mimic diabetic conditions where increased FFA oxidation contributes to inhibition of the PDH complex.

In contrast to inhibition of pyruvate decarboxylation by rac-LA reported previously,[14] our results show an increase in $^{14}CO_2$ production from [1-^{14}C]pyruvate in hepatocytes treated with R-LA (25 to 200 μmol/L; Fig 1). There are several experimental differences that may account for the discrepancy in results. First, the previous study[14] was performed with hepatocytes in suspension culture, which have a limited lifespan (6 hours) as compared to hepatocytes in monolayer culture. Short-term cultures of confluent monolayer hepatocytes cultured in WME are able to re-establish cell polarity, cell-cell contact, and communication, and demonstrate differentiated characteristics such as albumin secretion and expression of important metabolic enzymes and hormone receptors.[28] In addition, the suspension cultures were treated only during the $^{14}CO_2$ production assay (for 30 minutes), versus being pretreated for 3 hours prior to the assay in our monolayer cultures, which may affect the outcome of the study depending on the mechanism of the LA effect. This is also important in comparing the present study with studies performed using the purified PDH enzyme complex.[10,13] Interaction of LA with PDH in a cell-free system may be quite different from the intact cell environment considering concentrations of cofactors present and the presence or absence of the primary regulators of PDH complex activity, PDH-kinase, and PDH phosphatase, and of other molecules which may modify these reactions. Finally, in the hepatocyte suspension study, very high concentrations (1 mmol/L) of rac-LA were used. This may present 2 problems; first, at high concentrations the R- and S-enantiomers may differ significantly in regulatory activity,[10] although we found no evidence for this using 50 to 100 μmol/L R- and S-LA, and second, the high concentration may have been toxic to the cells, as we found that 3 hours exposure to R-LA concentrations 250 μmol/L and higher caused small but significant increases in LDH release in our monolayer cultures (data not shown).

Once we established that R-LA significantly increased pyruvate decarboxylation in our cultures, we wanted to determine if this increase reflected PDH complex activation. The percentage of active PDH did increase in R-LA–treated versus untreated cells (~2-fold with 50 μmol/L and ~3-fold increase with 100 μmol/L R-LA treatment) (Fig 2), suggesting that the drug promoted the dephosphorylation (activation) of the PDH complex, thereby increasing $^{14}CO_2$ production from [1-^{14}C]pyruvate. However, whether or not R-LA treatment stimulates hepatic pyruvate oxidation in vivo and increases PDH activity in liver of diabetic rats is yet to be demonstrated. Furthermore, although we did not observe major differences between R- and S-LA in stimulating pyruvate oxidation by rat hepatocytes in culture, differences between the 2 enantiomers may become more evident through in vivo studies in animal models and/or humans.

The reduction in glucose production from pyruvate following R-LA treatment (Fig 3) is supported by previous cell culture studies,[14] and by studies in control and diabetic rats.[23,29] The dramatic decrease (50% at 25 μmol/L and over 75% at 100 μmol/L R-LA) seen in gluconeogenesis in hepatocytes from fasted rats was reflected in lower total glucose concentration in the medium. The effect of R-LA on glucose production from pyruvate may reflect stimulation of glycolysis and/or inhibition of pyruvate carboxylase activation.

Another means by which LA could improve metabolism in the diabetic patient is through inhibition of FFA oxidation. Increased circulating FFAs and increased FFA oxidation in diabetes augment acetyl-CoA production, which in turn inhibits pyruvate and glucose oxidation and stimulates gluconeogenesis. Exposure of the hepatocytes to high concentrations of FFAs (1.5 mmol/L) increased palmitate oxidation nearly 9-fold compared to cells maintained at physiological concentrations of FFAs (0.1 mmol/L) (Fig 4). Treatment of the cells exposed to high FFAs with 25 μmol/L R-LA resulted in a 47% decrease in FFA oxidation. Higher concentrations of R-LA further decreased FFA oxidation in a dose-dependent manner, with a nearly 80% reduction in oxidation versus control at 200 μmol/L R-LA. Drug treatment caused small but significant reductions in palmitate oxidation even in 0.1 mmol/L FFA medium. It seems unlikely that R-LA simply competed for transport or metabolism with FFA because the inhibition of FFA oxidation by R-LA was much greater in the presence of high versus physiological concentrations of FFA in the medium. In rats treated in vivo with a high dose of rac-LA, plasma concentrations of free carnitine decreased while acylcarnitine concentrations increased, suggesting inhibition of acylcarnitine transport into the mitochondria or inhibition of carnitine acyltransferase-2 or of intramitochondrial FFA oxidation.[23] Sequestration of intramitochondrial CoA by LA has been suggested.[14,23]

The dramatic decrease in hepatic FFA oxidation, although potentially beneficial to overall glucose homeostasis, is of concern if the effect extends to other tissues, eg, skeletal and heart muscle, because it could aggravate the dislipidemia associated with diabetes. We therefore examined the effect of R-LA on FFA oxidation in normal (0.1 mmol/L) or high (1.0 mmol/L) FFA-containing medium in L6 myotube cultures. Interestingly, we found that FFA oxidation was unaffected in these cells in either medium by treatment with R-LA (Fig 5A). This indicates that R-LA–induced inhibition of FFA oxidation may be selective for certain tissues such as liver. The mechanism of this potential tissue specificity is unknown. It does not seem to involve limited transport of R-LA across the plasma membrane because R-LA stimulated pyruvate oxidation in L6 myotubes (Fig. 5B). In previous in vivo studies in Zucker rats, treatment with R-LA actually decreased circulating FFA concentrations[11]; however, short-term intravenous administration of a large dose of rac-LA (100 mg/kg) did increase circulating FFA in both fasted nondiabetic and diabetic rats.[23]

Two previous studies indicated that rac-LA decreased intramitochondrial free CoA levels in hepatocytes in suspension culture[14] and in liver in vivo,[23] possibly by trapping CoA as lipoyl-CoA. The concommitant reduction in acetyl-CoA concentration was 2.5- to 10-fold greater than the fall in free CoA in the 2 studies; thus the ratio of free CoA/acetyl-CoA increased markedly. A decrease in intramitochondrial free CoA may explain the marked inhibition of FFA oxidation observed here, and is consistent with previous studies.[23] The resulting fall in acetyl-CoA would explain the marked inhibition of gluconeogenesis from pyruvate, because acetyl-CoA serves as an activator of pyruvate decarboxylase. In addition the reduced availability of acetyl-CoA would decrease citrate synthesis, which in turn may accelerate glycolysis by activating phosphofructokinase.[14] CoA, a cofactor of the PDH complex, binds to the E_2 subunit with very high affinity. Thus, even a 50% reduction in free CoA would only minimally affect overall enzyme activity.[14,30] On the other hand, acetyl-CoA is a potent activator of PDH complex phosphorylation (deactivation). Thus, an increase in the ratio of free CoA/acetyl-CoA would result in activation of the PDH complex and increase pyruvate decarboxylation, which is consistent with our data in hepatocytes. However, in L6 myotubes, pyruvate oxidation was stimulated with no apparent impairment of FFA oxidation. Finally, LA is rapidly reduced to dihydro-LA (DHLA) in the cell, which in turn is released into the medium.[1,31,32] Thus, acting as an electron acceptor, R-LA may increase the intramitochondrial NAD/NADH ratio, and thereby promote PDH activation and flux through the PDH complex.

In summary, treatment of rat hepatocytes in primary culture with therapeutically relevant doses of R-LA for 3 hours increased pyruvate oxidation and activated the PDH complex. Concomitantly, glucose production from pyruvate and FFA oxidation were inhibited in a dose-dependent manner. Treatment of L6 myotubes with identical doses of R-LA did not affect FFA oxidation by these cells but increased pyruvate oxidation. Thus, while the antioxidant properties of R-LA may be effective in the prevention and/or treatment of diabetic complications, if confirmed in clinical studies, its ability to decrease hepatic glucose production may prove to be a useful adjunct in the treatment of type 2 diabetes.

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