□-Lipoic Acid is a Weak Dual PPAR□/□ Agonist

An Ester Derivative with Increased PPAR□/□ Efficacy and Antioxidant Activity

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KEY WORDS: antioxidant, insulin resistance, lipoic acid, lipoic acid ester, metabolic syndrome, oxidative stress, peroxisome proliferator-activated receptors, PPA R□, PPAR□, type 2 diabetes

ABSTRACT

□-Lipoic acid (LA), a potent antioxidant and free radical scavenger, has been reported to have insulin-sensitizing effects. In an attempt to improve the efficacy and antioxidant activity of LA, we synthesized and tested an ester derivative thereof: 6,8-bisacetylsulfanyl-octanoic acid ethyl ester (LA-ester). Both LA and LA-ester were shown to be weak dual PPAR□/□ agonists, and to modulate the expression of PPAR-regulated genes, promote adipogenesis, and reduce cellular oxidative stress. Of interest, LA-ester markedly increased the maximum activity of both PPAR□ and PPAR□, indicating increased efficacy. Both LA and LA-ester increased the expression of known PPAR □-regulated target genes (carnitine palmitoyltransferase 1A and acyl-CoA synthase) and PPAR□-regulated target genes (fatty acid translocase/CD36, adipocyte fatty acid-binding protein, lipoprotein lipase) all of which are key regulators of lipid and glucose metabolism. LA-ester had a markedly higher capacity for reducing

intracellular reactive oxygen species (cellular oxidative burden), compared to LA (30-fold vs. 2-fold reduction). Thus, LA, and perhaps to a greater extent LA-ester, may have insulin-sensitizing and possibly lipid-lowering effects, although further pre-clinical and clinical studies are required to confirm these claims. Esterification of the acidic moieties of LA may be a method for improving antioxidant activity and therapeutic efficacy of LA in the treatment of metabolic disorders known to be responsive to PPAR and PPAR ligands.

INTRODUCTION

Reactive oxygen species (ROS) or free radicals are generated during normal metabolism, physical exercise, exposure to chemotherapeutic agents, toxins and environmental (atmospheric) oxidants.[1,2] It has been proposed that excessive production of ROS leads to increased oxidative stress and activation of pro-inflammatory signaling pathways,thereby promoting and/or accelerating the pathology of aging and a variety of chronic metabolic diseases.[3-5] Therefore, the reduction of oxidative stress through administration of antioxidants could have a palliative or prophylactic effect on inflammatory, metabolic diseases, and even progression of the aging process.

-Lipoic acid (LA, thioctic acid, 5-(1,2-dithiolan-3-yl)pentanoic acid) occurs naturally in prokaryotic and eukaryotic cells and is an essential cofactor of the glycine cleavage system and the dihydrolipoamide acyltransferases of the pyruvate, -ketoglutarate and branched-chain -keto acid dehydrogenase complexes.[2] LA is a potent antioxidant and free radical scavenger, and its reduced form, dihydrolipoic acid (DHLA), has even greater antioxidant activity. The LA/DHLA oxidation/reduction couple has a redox potential greater than well-known antioxidant vitamins and cofactors, including -tocopherol (vitamin E), L-ascorbic acid (vitamin C), ubiquinone (coenzyme Q) and glutathione.[6]

LA has been shown to attenuate increases in mitochondrial superoxide anion production in the heart and advanced glycation end-product (AGE) content in the aorta; prevent reductions in tissue glutathione and glutathione peroxidase activity; and attenuate the hyperinsulinemia, hyperglycemia, and hypertension induced in Sprague-Dawley rats chronically fed glucose.[7,8] Treatment with LA prevented fructose-induced[9,10] and salt-induced[11] increases in blood pressure in rats and suppressed increases in blood pressure in the spontaneously hypertensive rat.[12] Furthermore, LA corrected cardiac lipid abnormalities and biomarkers of oxidative stress,[13] and ameliorated metabolic parameters, including blood pressure, vascular reactivity and morphology of damaged vessels in streptozotocin-induced diabetes in rats.[14]

Treatment with LA significantly decreased plasma lipid hydroperoxides, a composite marker of oxidative stress and repair antioxidant defense mechanisms in diabetic patients,[15] and improved insulin-stimulated glucose disposal[16] and insulin sensitivity in patients with type 2 diabetes.[17] Therefore, it has been proposed that because of its ability to reduce oxidative stress, and oppose the increased oxidative stress and depleted antioxidant defense in diabetes, LA may have insulin-sensitizing effects,[18,19] and may help prevent diabetes complications in humans.[20,21]

The peroxisome proliferator activated receptors (PPAR ,,*∂*) are transcription factors that modulate key genes in glucose and lipid intermediary metabolism. The discovery that the thiazolidinedione (TZD) class of compounds were high affinity agonists for PPAR, and that key genes involved in glucose and lipid metabolism possess PPAR gene

response elements, led to the proposal that ligand-dependent modulation of gene transcription through PPARγ activation was an important mechanism in the treatment of diabetes.[22] Rosiglitazone and pioglitazone are insulin-sensitizing TZDs presently in clinical use for the treatment of type 2 diabetes. Fenofibrate and gemfibrozil, triglyceride-lowering drugs also in clinical use, were discovered to activate PPARα and shown to modulate the expression of genes involved in fatty acid oxidation, and triglyceride and lipoprotein metabolism.[23]

Because PPARγ ligands are proven insulin-sensitizing agents, and LA was shown to have insulin-sensitizing effects in animal models of insulin resistance, we set out to determine if LA could activate PPARs.

Moreover, in an attempt to improve upon the PPAR actions of LA, we synthesized ethyl 6,8-bis(ethanethio)-octanoate ester (LA-ester) derivative of LA (Figure 1). Herein we report the effects of LA and LA-ester on PPARα and PPARγ transactivation, induction of adipogenesis, target gene expression, and their capacity for reducing intracellular oxidative stress.

MATERIALS AND METHODS

Materials

LA and all other materials were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO). Racemic mixtures of LA and LA-ester were used in all experiments. LA-ester was synthesized from commercially available enantiomeric (DL)-LA, and purity confirmed by infra red, nuclear magnetic resonance and mass spectroscopy.

Cell Culture

Murine 3T3-L1 preadipocytes (CCL-173) and CV-1 monkey epithelial cells (CCL-70) were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) (3T3-L1) or 5% Cosmic Calf Serum (CV-1) (Hyclone, Logan, UT). The HepG2 human hepatoma-derived cell line stably expressing elevated levels of transfected human PPARγ was provided by Dr. Eric Johnson from the Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA.[24] These cells were maintained in DMEM, 10% fetal bovine serum, 500 µg/mL Geneticin (G418).

PPAR Transactivation Assays

Ligand activated PPAR activity was determined by a cell-based transactivation assay utilizing CV-1 cells as described previously.[25] Transfections were performed using the GenePorter (San Diego, CA) reagent according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with the ligand diluted in DMEM, 0.5% charcoal-dextran treated FBS and incubated for an additional 24 hours. The cells were washed twice with phosphate buffered saline (PBS) and assayed for luciferase and β-galactosidase activity using the Promega luciferase and β-galactosidase assay systems according to the manufacturer's instruction. All treatments were performed in triplicate, and luciferase activity was normalized for β-galactosidase activity. Concentrations of ligand-yielding half-maximal activation (EC50 values) were calculated using curve-fitting software (GraphPad Prism, San Diego, CA).

Gene Expression Assays

For determination of PPARγ-regulated gene expression, confluent 3T3-L1 preadipocytes were treated with differentiation cocktail containing dexamethasone, insulin and 1-methyl-3-isobutylxanthine for 28 hours

as described for the adipocyte differentiation assay. The cells were then incubated in medium containing the test compound or vehicle, DMSO (dimethyl sulfoxide) for 48 hours. RNA was isolated using the RNAqueous-4PCR kit (Ambion, Austin, TX) including the DNase treatment. Quantitative Real-time PCR (qRTPCR) was used to measure target gene expression. The one-step SYBR Green Quantitative RT-PCR Kit (Sigma, St. Louis, MO) was used in an Opticon II continuous fluorescence detector. The cyclophilin (peptidylprolyl isomerase A) gene was used as an internal control for normalization with results being determined in triplicate. Fold changes in gene expression are displayed as the amount of normalized mRNA in LA- and LA-ester-treated samples relative to that in the vehicle-treated controls, which was arbitrarily defined as 1.

For determination of PPAR□-regulated gene expression, confluent HepG2 cells[26] were treated with test compound or vehicle followed by RNA isolation and target gene expression as described above.

Adipocyte Differentiation Assay

Differentiation assays on preadipocytes were performed by a modification of the technique described by Smith et al.[27] In brief, murine 3T3-L1 cells were plated in DMEM with 5% FBS and grown to confluence. After reaching confluence, cells were incubated in DMEM containing 1.0 µmol/L dexamethasone, 5 µg/mL insulin and 0.5 mmol/L 1-methyl-3-isobutylxanthine with 5% FBS for 28 hours, after which cells were washed with PBS and incubated in medium containing the test compound or vehicle, DMSO (dimethyl sulfoxide). Five days after treatment, cells were fixed with 10% formalin in PBS and stained with Oil Red O.

Oxidative Stress and Cell Viability

Mouse embryonic fibroblasts with targeted deletions in both Nrf1 and Nrf2 genes that exhibit marked oxidative stress[28] were used to test the antioxidant effects of LA and LA-ester. These double knockout cells were cultured in DMEM with 10% FBS, 2mM L-glutamine, 1mM non-essential amino acids, and 0.05 mM 2-mercaptoethanol supplemented with LA and LA-ester at the indicated concentrations. Intracellular ROS levels were assessed using the oxidation-sensitive fluorescent probe (2´,7´-dichlorodihydrofluorescein-diacetate) (DCFH-DA). The double knockout cells were incubated with either LA and LA-ester at the indicated concentrations for 12 hours prior to loading with 10 µM DCFH-DA. After 30 minutes of incubation, cells were harvested**,** washed once and resuspended in PBS containing 0.5% FBS Propidium iodide (1 µg/mL), was added prior to analysis and was used to gate out dead cells. The oxidative conversion of DCFH-DA to fluorescent products was assessed in live cells by flow cytometry.

RESULTS

Structures of LA and LA-ester

The chemical structures of LA and the LA-ester, each containing a single chiral carbon, are shown in Figure 1. Racemic mixtures of both compounds were used in all studies. The ester bonds shown are hydrolysable by non-specific esterases under physiological conditions.

LA and LA-ester Activate PPAR□ and PPAR□

Activation of PPAR□by LA and LA-ester was shown to be concentration-dependent (Figure 2A). At 0.5 mM, LA increased PPAR□/GAL4 fusion protein activity 20-fold greater than that seen with vehicle-treated cells. At 2 mM, LA and LA-ester increased PPAR□activity by 260-fold and 600-fold, respectively,

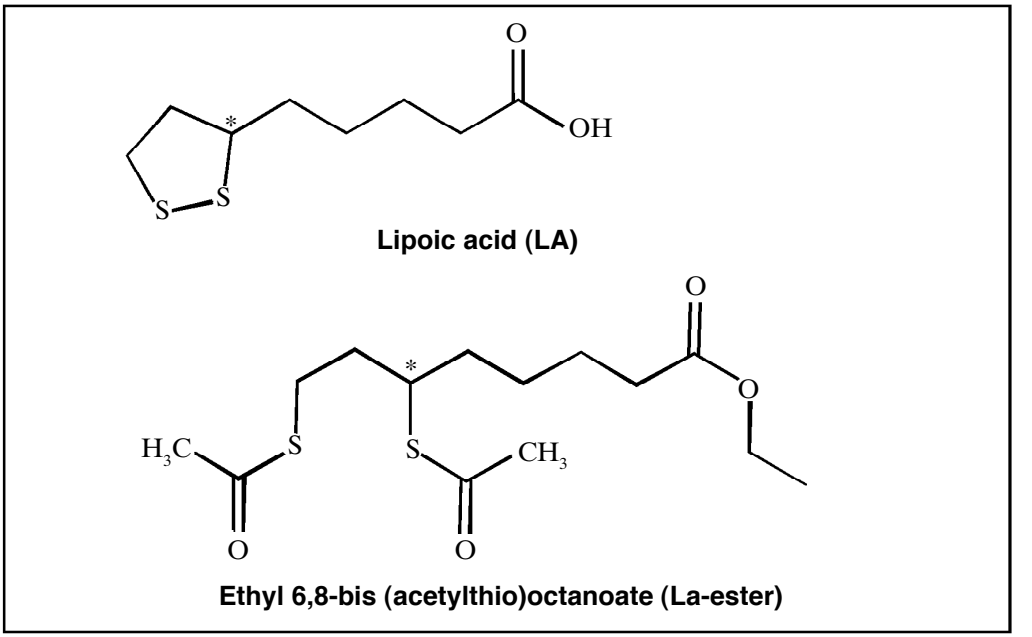

Figure 1. Structural formulae of □-lipoic acid (LA) and LA-ester. Each containing a single chiral carbon (*).

indicating greater efficacy of the LA-ester compared to LA. Both compounds had narrow activation thresholds with similar EC50 values: 0.98 mM for LA and 1.04 mM for LA-ester (Figure 2B). Activation of PPAR□ by LA and LA-ester was also shown to be concentration-dependent (Figure 3A). Again, maximum activation of the PPAR□/GAL4 fusion protein by LA-ester was greater than that of LA. Between 0.5 and 4 mM, LA increased the activity of PPAR□ by 3- to 30-fold, whereas LA-ester increased PPAR□ activity from 6- to 110-fold. Activation thresholds for both compounds were again similar, with similar EC50 values: 2.1 mM for LA and 2.7 mM for LA-ester (Figure 3B). Neither compound had any effect on PPARd activity (data not shown).

LA and LA-Ester Induce the Expression of PPAR□ and PPAR□ Target Genes

Since LA and LA-ester functioned as dual PPAR□/□agonists, we investigated whether these ligands would induce the expression of target genes modulated by these receptors. HepG2 human hepatocarcinoma cells engineered to stably express PPAR□ at concentrations found in vivo were used to determine the effects of these compounds on PPAR□-regulated target genes. Confluent cultures were exposed for 48 hr to either vehicle (DMSO), LA, LA-ester, or WY-14643 (positive control), a known PPAR□ agonist. Compared to DMSO, LA (2 mM) increased the expression of carnitine palmitoyltransferase 1A (CPT1A) and acyl-CoA synthase (ACS), by 3-fold and 7-fold, respectively (Figure 4A). Similarly, LA-ester (2 mM) increased the expression of CPT1A and ACS by 4-fold and 5-fold, respectively (Figure 4A). These data indicate that LA and LA-ester regulated the endogeneous promoter region of two known PPAR□ responsive genes.

To investigate the effects of LA and LA-ester on PPAR□regulated target

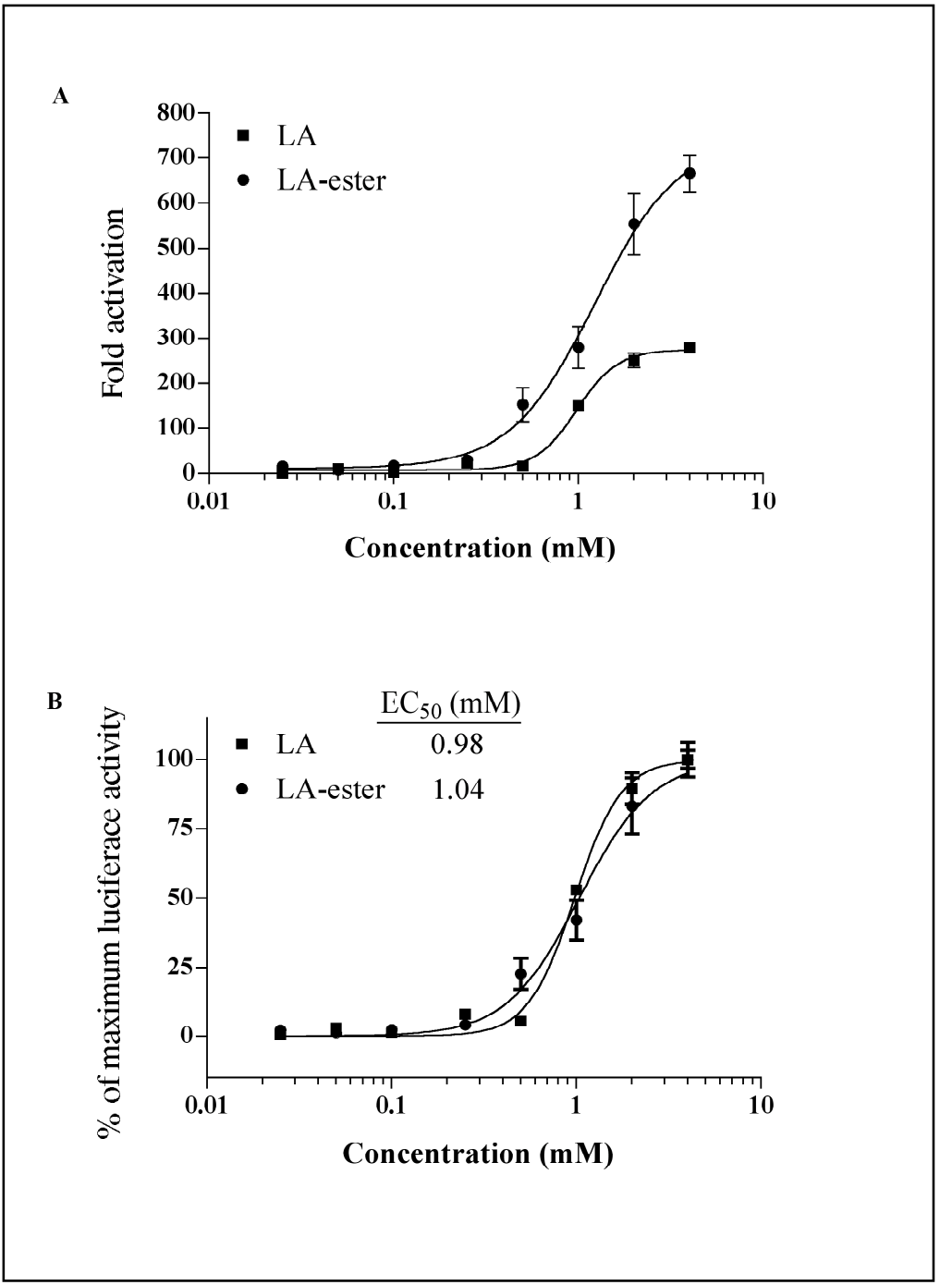

Figure 2. Activation profile of PPAR by LA and LA-ester. Transactivation of the GAL4-PPAR chimeric receptor by LA and LA-ester in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hr and normalized total luciferase activity determined. (A) Fold activation as compared to vehicle treatment as a function of ligand concentration. (B) Data from Figure 2A presented as percent of maximal activity as a function of ligand concentration. LA (square), LA-ester (circle).

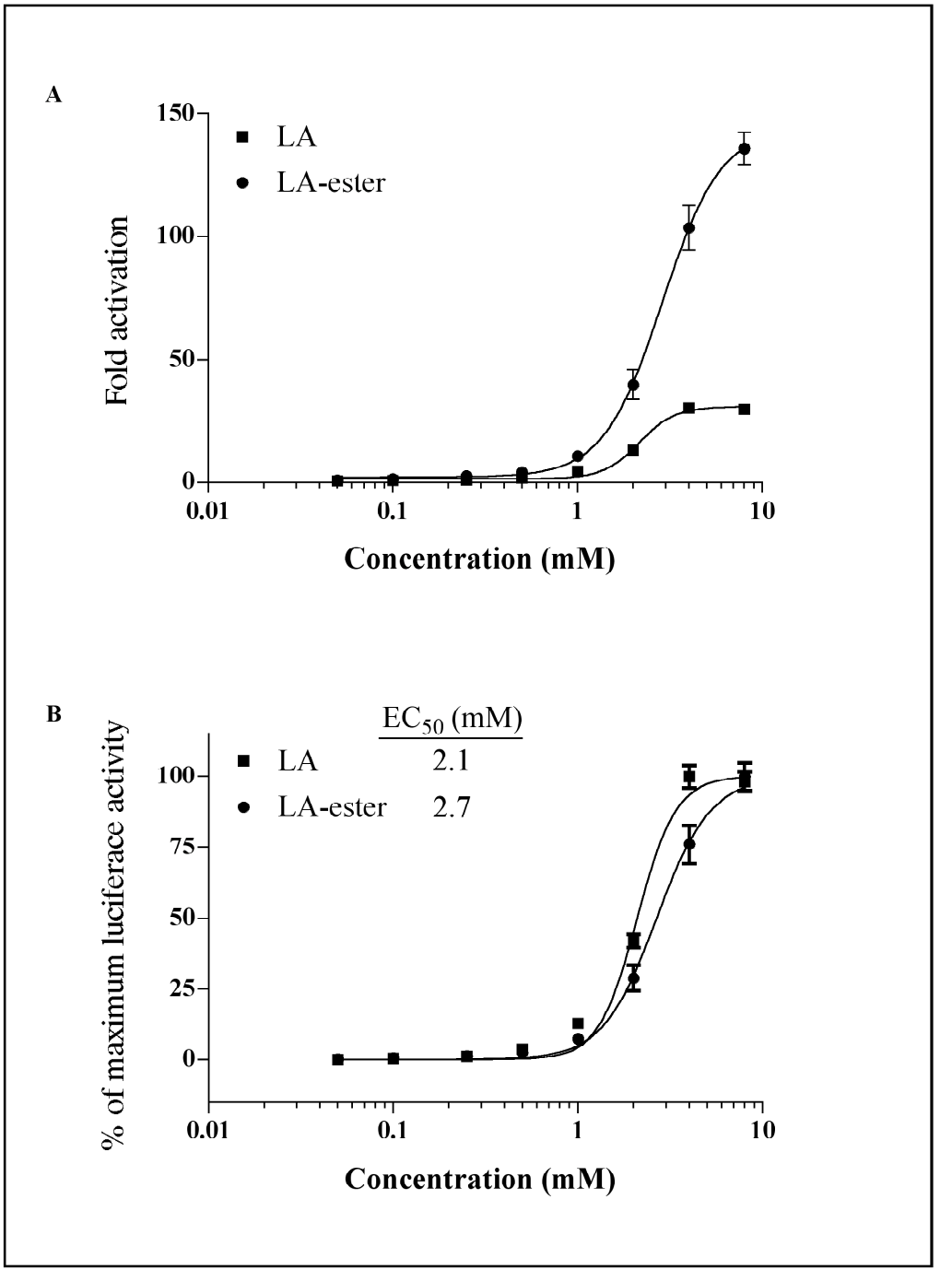

Figure 3. Activation profile of PPAR□ by LA and LA-ester. Transactivation of the GAL4-PPAR□ chimeric receptor by LA and LA-ester in transiently transfected CV-1 cells. Transfected cells were treated with the indicated concentrations of ligand for 24 hr and normalized total luciferase activity determined. (A) Fold activation as compared to vehicle treatment as a function of ligand concentration. (B) Data from Figure 3A presented as percent of maximal activity as a function of ligand concentration. LA (square), LA-ester (circle).

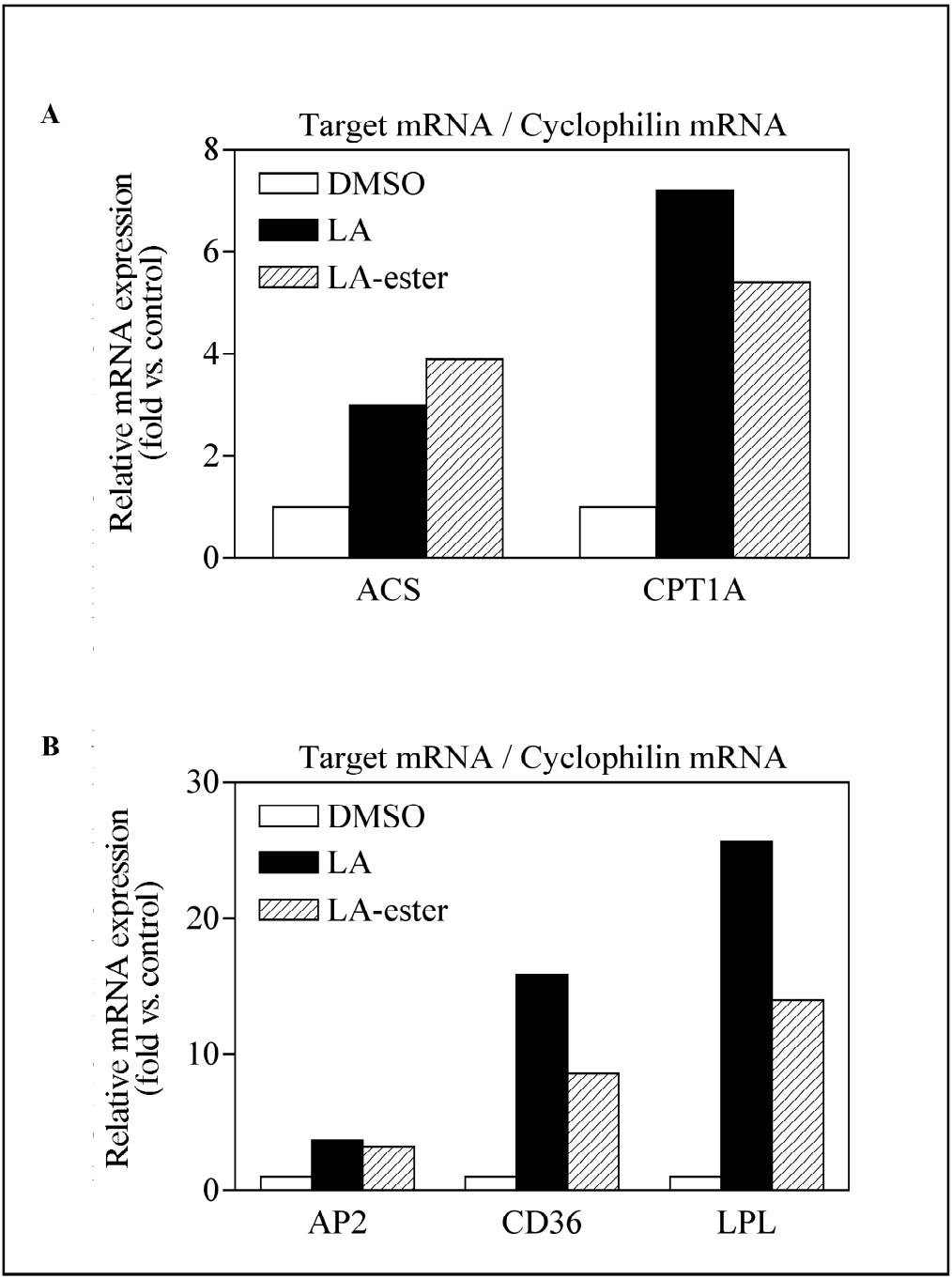

Figure 4. Induction of PPAR□ and PPAR□target gene expression by LA and LA-ester. (A) PPAR□ target genes: epG2 cells were treated with LA (2 mM), LA-ester (2 mM), or vehicle (DMSO) for 48 hr and the expression of PPAR□ target genes ACS and CPT1A were determined by qRT-PCR. (B) PPAR□target genes: 3T3-L1 preadipocytes were treated with 2mM LA (2 mM), LA-ester (2 mM), or vehicle (DMSO) for 48 hr and the expression of PPAR□target genes AP2, CD36, and LPL were determined by qRT-PCR. RNA levels for all target genes were normalized to levels of cyclophilin mRNA.

genes, murine 3T3-L1 preadipocytes were induced to differentiate by exposure to differentiation medium for 2 hr, then for 48 hr to vehicle (DMSO), LA, LA-ester or rosiglitazone (positive control), a known PPARγ agonist. The effect of LA and LA-ester on the expression of fatty acid translocase/CD36 (CD36), adipocyte fatty acid-binding protein (AP2), and lipoprotein lipase (LPL) is shown in Figure 4B. Rank order increase in the expression of these 3 genes upon exposure to LA were: LPL (26-fold) > CD36 (16-fold) > AP2 (3-fold), and upon exposure to LA-ester were: LPL (14-fold) > CD36 (8-fold) > AP2 (2.5-fold) (Figure 4B). Both compounds were most effective in increasing the expression of LPL and CD36, with only a modest effect on AP2 expression. These data confirm that LA and the LA-ester can increase the expression of at least three PPARγ-regulated genes.

LA and LA-ester Can Induce Differentiation of 3T3L1 Preadipocytes

The hallmark property of PPARγ agonists is their ability to induce differentiation and maturation of preadipocytes.[22] Therefore, we investigated whether LA and LA-ester could promote adipogenesis in 3T3-L1 preadipocyte fibroblasts. Both LA and LA-ester induced adipogenesis as shown by Oil Red O staining in 3T3-L1 cells, when tested at concentrations 0.5 to 2 mM. The effects of 0.5 mM LA and 0.5mM LAester on adipogenesis are shown in Figure 5B and C, respectively.

LA-Ester Provides Enhanced Protection against Oxidative Stress

Double knockout mouse embryonic fibroblasts with targeted deletions in both Nrf1 and Nrf2 are highly susceptible to cellular death due to oxidative stress.[28] Intracellular ROS levels were assessed using 2´,7´-dichlorodihydrofluorescein diacetate (DCFH-DA) ester.

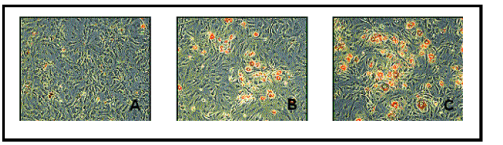

Figure 5. Induction of adipogenesis by LA and LA-ester. Confluent cultures of 3T3-L1 murine preadipocytes were treated with insulin, dexamethasone and IBMX for 32 hr. The cells were then treated with vehicle (DMSO) or LA (0.5 mM), or LA-ester (0.5 mM), for 5 days. Following fixation with 10% formalin in PBS, the cells were stained with Oil Red O. (A) Vehicle (0.004% v/v DMSO); (B) LA (C) LA-ester.

DCFA-DA is accumulated intracellularly where it is oxidized by ROS inside cells to the highly fluorescent 2´,7´-dichlorofluorescein (DCFH) acid by intracellular ROS. Therefore, the fluorescent intensity of DCFH-DA oxidation products collectively trapped intracellularly is an indicator of intracellular ROS. Control (untreated) cells had a mean fluorescence of 780 (arbitrary units) (Figure 6A). When exposed to 0.5 mM LA for 12 hr, the fluorescent intensity of live cells decreased almost 2-fold, to a mean fluorescence of 414 units (Figure 6B), indicating a reduction of cellular ROS. In contrast, cells exposed to 0.5 mM LA-ester for 12 hr had a 30-fold reduction in mean fluorescence of 26 units (Figure 6C), indicating that LA-ester was 15 times better than LA at reducing the intracellular ROS and oxidative stress.

DISCUSSION

In this study, we investigated whether the potent antioxidant and free radical scavenger LA activated PPARs and whether the prodrug, LA-ester (Figure 1) might improve PPAR activity or efficacy (maximal activation) and modulate the expression of PPAR-regulated target genes. In artificial chimeric PPAR-Gal4 receptor assays, both LA and LA-ester were found to be weak dual PPARγ/δ agonists with similar activation profiles, whereas LA-ester had

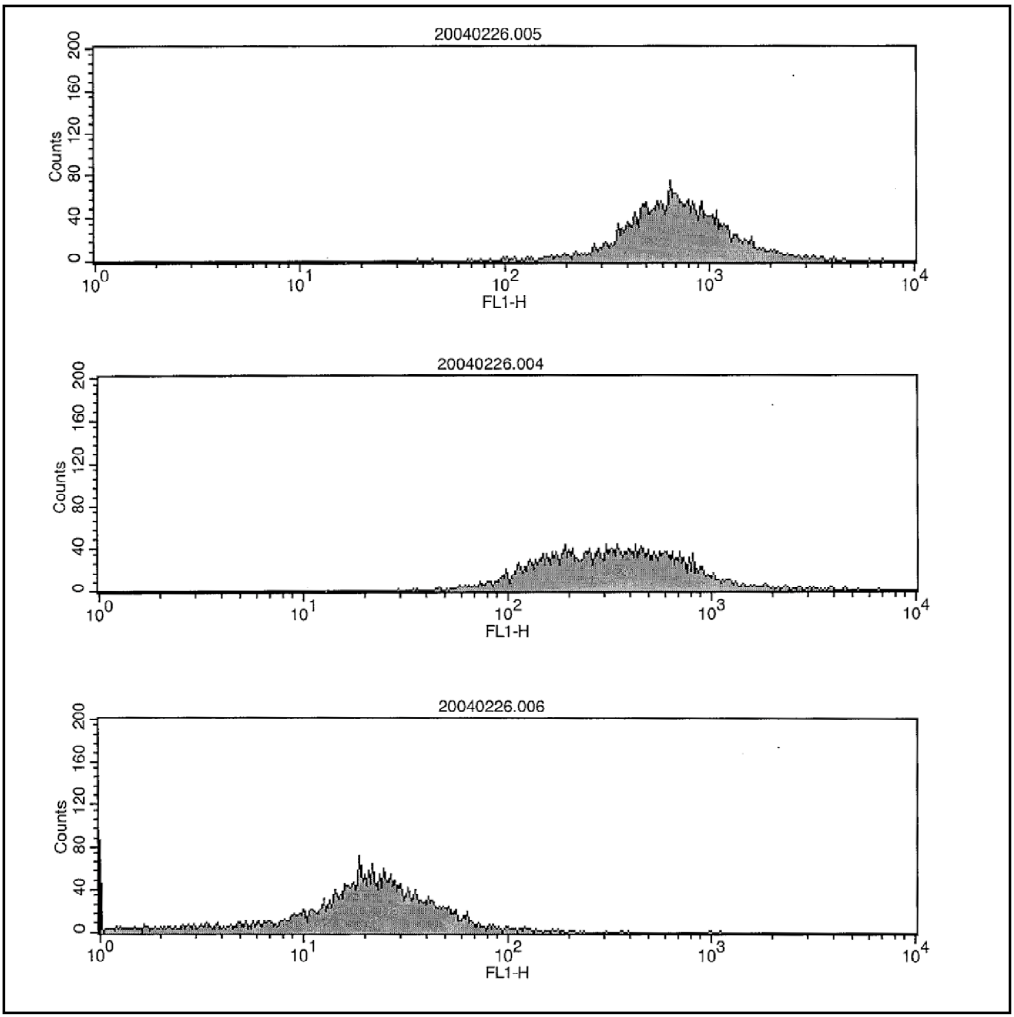

Figure 6. Reduction of intracellular ROS levels by LA and LA-ester. Embryonic fibroblasts derived from Nrf1/Nrf2 knockout mice were loaded with the non-fluorescent dye 2′,7′-dichlorodihydrofluorescein acetate and formation of ROS was measured by monitoring the fluorescent intensity of dichlorofluorescein, shown on the abscissa (log scale). Cells treated with vehicle (DMSO) treated is shown in upper panel (A). Cells treated with 0.5 mM LA and 0.5 mM LA-ester are shown in middlepanel (B), and lower panel (C), respectively.

greater efficacy in activating PPAR and PPAR over the same concentration range (Figures 2 and 3). Both compounds similarly increased the expression of PPAR-regulated target genes in hepatocytes (Figure 4). Regarding PPAR-regulated target genes, both LA and LA-ester increased the expression of AP2, though the latter compound had a decreased effect on CD36 and LPL in adipocytes (Figure 4). Both LA and LA-ester similarly induced adipogenesis and promoted adipocyte differentiation, characterized by the intracellular accumulation of lipids (Figure 5). This is consistent with previous observations, that ligand-dependent PPAR activation triggers the genetic program for adipocyte differentiation and maturation, a signature effect of PPAR agonists.[22,29] Neither LA nor LA-ester activated PPAR∂. Finally, compared to LA, LA-ester had a markedly higher antioxidant capacity. Exposure of cells to LA-ester

(0.5 mM) had a 30-fold reduction in cellular burden of ROS compared to a 2-fold reduction in cells exposed to LA (also 0.5 mM), indicating that LA-ester provided enhanced protection of against cellular oxidative stress, in vivo.

Esterification of ionized species is a widely employed method of increasing cellular permeability. Once inside cells, the ester linkage(s) are exposed to enzymatic hydrolysis by esterases to generate the corresponding acid. Because an acidic group is essential for stabilizing PPAR ligands within the PPAR receptor ligand-binding domain,[22] one may hypothesize that at least one of the hydrolysable groups of LA-ester, probably the ethyl ester, would have been cleaved to cause the molecule to activate PPAR and PPAR. Regardless, the mechanism whereby esterification improves efficacy in PPAR-Gal4 receptor assays and increases cellular protection against oxidative stress requires further study.

At pharmacological concentrations, LA has been reported to have a marginal beneficial effect in humans with type 2 diabetes.[18,19,30] Intravenous infusion of LA into type 2 diabetic patients was shown to increase insulin-stimulated glucose disposal and insulin sensitivity by 55% and 57%, respectively.[16,17] To be effective orally, LA would have to be administered at pharmacological concentrations, requiring it to be formulated in such a way (eg, as a slow release preparation) as to achieve sustained plasma concentrations required for antidiabetic activity.[31] Thus, if the limitations of oral administration can be overcome, LA could achieve antidiabetic efficacy. Our study implies that specially designed esters of LA may possibly be a rational approach for improving its therapeutic efficacy. Future studies in animal models of insulin resistance and/or type 2 diabetes are needed to evaluate the pharmacokinetic profile and efficacy of various LA esters. Because PPAR and PPAR agonists have been shown to lower triglycerides[22,23] and suppress pro-inflammatory markers,[32-34] it may also be possible to design optimized LA-ester derivatives to improve their metabolic benefits.[18,31]

The metabolic syndrome is a recently recognized cluster of high cardiovascular risk factors comprising insulin resistance, visceral obesity, elevated triglycerides, low HDL-cholesterol, and elevated blood pressure.[35,36] Because PPAR/ agonists can have the combined effects of increasing insulin sensitivity, lowering blood pressure, improving the lipid profile and potentially mitigating atherosclerosis, these ligands are potential candidates for treating the metabolic syndrome.[23,32-34,37] Therefore, it may be possible to design esters of LA to improve their dual PPAR/ activity, with increased efficacy of treating type 2 diabetes and the metabolic syndrome.

The finding that LA and LA-ester can function as a dual PPAR/ activator may, at least in part, provide a mechanistic rationale for observed insulin-sensitizing and other beneficial effects of LA. Esterification may provide a platform for the rational development of a library of esters of LA for treating a variety of metabolic diseases. Moreover, by reducing oxidative stress, such molecules may reduce systemic inflammation, improve endothelial function and oppose pro-atherogenic processes. LA has previously been reported to function as a weak PPAR activator that inhibited PPAR transactivation induced by the TZD, troglitazone,[38] consistent with the speculation that LA may function as a partial PPAR agonist.

Of particular interest are the recent findings that oral administration of LA to rodents reduced body weight by reducing food intake and enhancing

energy expenditure by suppressing hypothalamic AMP kinase activity,[39] and that LA improved insulin sensitivity by increasing fatty acid oxidation and lowering triglyceride accumulation in skeletal muscle by activating AMP kinase.[40] These pathways have been proposed as mechanisms whereby the antidiabetic drug, metformin, and to some extent PPAR agonists, may evoke their insulin-sensitizing efficacy in the treatment for type 2 diabetes.[41-43] Therefore, the effects of LA-ester on body weight, tissue AMP kinase activities, skeletal muscle fatty acid oxidation and triglyceride accumulation, warrant exploration.

In conclusion, the findings reported herein provide a rationale for developing ester derivatives of LA with improved efficacy for the treatment of type 2 diabetes and the metabolic syndrome.

ACKNOWLEDGEMENTS

Supported by NIH Grant 2R42AR44767-02A2 (H.A.P.), a Joint Venture Grant from California State University Program for Education and Research in Biotechnology (S.C.B.), and a grant from Bethesda Pharmaceuticals, Inc.

DISCLOSURE STATEMENT

H. A. Pershadsingh and T. W. Kurtz are principals in and own stock in Bethesda Pharmaceuticals, Inc., which holds intellectual property related to the synthesis and use of novel lipoic acid derivatives.

REFERENCES

1. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr.* 1996;16:33-50.
2. Biewenga GP, Haenen GR, Bast A. The pharmacology of the antioxidant lipoic acid. *Gen Pharmacol*. 1997;29:315-331.
3. Winrow VR, Winyard PG, Morris CJ, Blake DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br Med Bull.* 1993;49:506-522.
4. Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. *FASEB J*. 1996;10:709-720.
5. Liu J, Atamna H, Kuratsune H, Ames BN. Delaying brain mitochondrial decay and aging with mitochondrial antioxidants and metabolites. *Ann NY Acad Sci*. 2002;959:133-166.
6. Podda M, Grundmann-Kollmann M. Low molecular weight antioxidants and their role in skin ageing. *Clin Exp Dermatol*. 2001;26:578-582.
7. El Midaoui A, de Champlain J. Prevention of hypertension, insulin resistance, and oxidative stress by alpha-lipoic acid. *Hypertension.* 2002;39:303-307.
8. Midaoui AE, Elimadi A, Wu L, Haddad PS, de Champlain J. Lipoic acid prevents hypertension, hyperglycemia, and the increase in heart mitochondrial superoxide production. *Am J Hypertens*. 2003;16:173-179.
9. Vasdev S, Ford CA, Parai S, et al. Dietary lipoic acid supplementation prevents fructose-induced hypertension in rats. *Nutr Metab Cardiovasc Dis*. 2000;10:339-346.
10. Thirunavukkarasu V, Anitha Nandhini AT, Anuradha CV. Lipoic acid attenuates hypertension and improves insulin sensitivity, kallikrein activity and nitrite levels in high fructose-fed rats. *J Comp Physiol. [B]*. 2004;174:587-592.
11. Vasdev S, Gill V, Longerich L, et al. Salt-induced hypertension in WKY rats: prevention by alpha-lipoic acid supplementation. *Mol Cell Biochem*. 2003;254:319-326.
12. Vasdev S, Ford CA, Parai S, et al. Dietary alpha-lipoic acid supplementation lowers blood pressure in spontaneously hypertensive rats. *J Hypertens*. 2000;18:567-573.
13. Thirunavukkarasu V, Anitha Nandhini AT, Anuradha CV. Cardiac lipids and antioxidant status in high fructose rats and the effect of alpha-lipoic acid. *Nutr Metab Cardiovasc Dis*. 2004;14:351-357.
14. Kocak G, Aktan F, Canbolat O, et al. ADIC Study Group—Antioxidants in Diabetes-Induced Complications. Alpha-lipoic acid treatment ameliorates metabolic parameters, blood pressure, vascular reactivity and morphology of vessels already damaged by streptozotocindiabetes. *Diabetes Nutr Metab*. 2000;13:308-318.
15. Borcea V, Nourooz-Zadeh J, Wolff SP, et al. -Lipoic acid decreases oxidative stress even in diabetic patients with poor glycemic control

and albuminuria. *Free Radic Biol Med*. 1999;26:1495-1500.

16. Jacob S, Henriksen EJ, Tritschler HJ, et al. Improvement of insulin-stimulated glucose-disposal in type 2 diabetes after repeated parenteral administration of thioctic acid. *Exp Clin Endocrinol Diabetes*. 1996;104:284-288.

17. Jacob S, Ruus P, Hermann R, et al. Oral administration of RAC-alpha-lipoic acid modulates insulin sensitivity in patients with type-2 diabetes mellitus: a placebo-controlled pilot trial. *Free Radic Biol Med*. 1999;27:309-314.

18. Evans JL, Goldfine ID. a-lipoic acid: a multifunctional antioxidant that improves insulin sensitivity in patients with type 2 diabetes. *Diabetes Technol Ther*. 2000;2:401-413.

19. Rosen P, Nawroth PP, King G, Moller W, et al. The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society. *Diabetes Metab Res Rev*. 2001;17:189-212.

20. Packer L, Kraemer K, Rimbach G. Molecular aspects of lipoic acid in the prevention of diabetes complications. *Nutrition*. 2001;17:888-895.

21. Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? *Circulation*. 2002;105:2107-2111.

22. Willson TM, Lambert MH, Kliewer SA. Peroxisome proliferator-activated receptor gamma and metabolic disease. *Annu Rev Biochem*. 2001;70:341-367.

23. Staels B, Dallongeville J, Auwerx J, et al. Mechanism of action of fibrates on lipid and lipoprotein metabolism. *Circulation.* 1998;98:2088-2093.

24. Hsu MH, Savas U, Griffin KJ, Johnson EF. Identification of peroxisome proliferator-responsive human genes by elevated expression of the peroxisome proliferator-activated receptor alpha in HepG2 cells. *J Biol Chem.* 2001;276:27950- 27958.

25. Benson SC, Pershadsingh HA, Ho CI, et al. Identification of telmisartan as a unique angiotensin II receptor antagonist with selective PPARg-modulating activity. *Hypertension.* 2004;43;993-1002.

26. Vanden Heuvel JP, Kreder D, Belda B, et al. Comprehensive analysis of gene expression in rat and human hepatoma cells exposed to the peroxisome proliferator WY14,643. *Toxicol Appl Pharmacol*. 2003;188:185-198.

27. Smith PJ, Wise LS, Berkowitz R, et al. Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J Biol Chem.* 1988;263:9402-9408.

28. Leung M, Kwong S, Hou S, et al. Deficiency of the Nrf1 and Nrf2 transcription factors results in early embryonic lethality and severe oxidative stress. *J Biol Chem*. 2003;278:48021-48029.

29. Lehmann JM, Moore LB, Smith-Oliver TA, et al. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem.* 1995;270;12953-12956.

30. Konrad T, Vicini P, Kusterer K, et al. □-Lipoic acid treatment decreases serum lactate and pyruvate concentrations and improves glucose effectiveness in lean and obese patients with type 2 diabetes. *Diabetes Care.* 1999;22:280-287.

31. Evans JL, Heymann CJ, Goldfine ID, Gavin LA. Pharmacokinetics, tolerability, and fructosamine-lowering effect of a novel, controlled-release formulation of alpha-lipoic acid. *Endocr Pract.* 2002;8:29-35.

32. Debril MB, Renaud JP, Fajas L, Auwerx J. The pleiotropic functions of peroxisome proliferator-activated receptor-□ *J Mol Med.* 2001;79:30-47.

33. Delerive P, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors in inflammation control. *J Endocrinol*. 2001;169:453-459.

34. Pershadsingh HA. Peroxisome proliferator-activated receptor-□ therapeutic target for diseases beyond diabetes: quo vadis? *Expert Opin Investig Drugs.* 2004;13:215-228.

35. NCEP/ATP III: Executive Summary of the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA*. 2001;285:2486–2497.

36. Lakka HM, Laaksonen DE, Lakka TA, et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *JAMA*. 2002;288:2709-2716.

37. Lebovitz HE, Banerji MA. Insulin resistance and its treatment by thiazolidinediones. *Recent Prog Horm Res.* 2001;56:265-294.

38. Cho KJ, Moini H, Shon HK, et al. a-Lipoic acid decreases thiol reactivity of the insulin receptor and protein tyrosine phosphatase 1B in 3T3- L1 adipocytes. *Biochem Pharmacol.* 2003;66:849-858.

39. Kim MS, Park JY, Namkoong C, et al. Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med*. 2004;10:727-33.

40. Lee WJ, Song KH, Koh EH, et al. alpha-Lipoic acid increases insulin sensitivity by activating AMPK in skeletal muscle. *Biochem Biophys Res Commun*. 2005; 332:885-91.

41. Zhou G, Myers R, Li Y, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* 2001;108:1167-1174.

42. Musi N, Hirshman MF, Nygren J, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes*. 2002;51:2074-2081.

43. Hardie DG. Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology.* 2003;144:5179-5183.