

# Zinc Deficiency Alters Lipid Metabolism in LDL Receptor-Deficient Mice Treated with Rosiglitazone<sup>1,2</sup>

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## Abstract

Zinc is a structural and functional component of PPAR and zinc deficiency may be associated with an increased risk for cardiovascular diseases. We tested the hypothesis that zinc deficiency compromises lipid metabolism in rosiglitazone (RSG)-treated mice lacking the LDL-receptor (LDL-R) gene. LDL-R-deficient mice were maintained for 3 wk on low-fat (7 g/100 g) diets that were either zinc deficient or zinc adequate. Subsequently, diets were adjusted to a high-fat (HF) (15 g/100 g) regimen for 1 wk to produce a biological environment of mild oxidative and inflammatory stress. One-half of the mice within each zinc group was gavaged daily with the PPAR $\gamma$  agonist RSG starting 2 d prior to the HF feeding. Selected lipid parameters were studied. Zinc deficiency increased plasma total cholesterol, which was also elevated by RSG. Zinc deficiency also caused an increased lipoprotein-cholesterol distribution toward the non-HDL fraction (VLDL, intermediate density lipoprotein, LDL). Plasma total fatty acids tended to increase during zinc deficiency and RSG treatment resulted in similar changes in the fatty acid profile in zinc-deficient mice. Fatty acid translocase (FAT/CD36) expression in abdominal aorta was upregulated by RSG only in zinc-deficient mice. In contrast, RSG treatment markedly increased lipoprotein lipase (LPL) expression only in zinc-adequate mice. In vitro studies confirmed that adequate zinc is required for RSG-induced PPAR $\gamma$  activity to transactivate target genes. These data suggest that in this atherogenic mouse model treated with RSG, lipid metabolism can be compromised during zinc deficiency and that adequate dietary zinc may be considered during therapy with the antidiabetic medicine RSG. *J. Nutr.* 137: 2339–2345, 2007.

## Introduction

Cardiovascular diseases are a major health problem in industrialized countries and have an increasing incidence in the nonindustrialized part of the world as well. Causes for the development of atherosclerosis are usually multiple. Lifestyle and nutrition can be closely linked to the onset and the pace of progression of atherosclerotic events (1,2). Hyperlipidemia, central obesity, impaired glucose tolerance, and overall insulin resistance are among the many risk factors associated with accelerated pathology of atherosclerosis (3).

Studies in rodent models suggest that zinc supplementation is effective for reducing the incidence of both type 1 and type 2 diabetes (4) and that zinc deficiency can activate stress pathways resulting in loss of insulin sensitivity (5). Evidence also suggests

that type 2 diabetic patients experience zinc malabsorption and increased excretion of urinary zinc (6).

Synthetic PPAR $\gamma$  agonists, such as thiazolidinediones [including rosiglitazone (RSG) and pioglitazone], improve insulin sensitivity and glycemic control in type 2 diabetes and may reduce atherosclerosis progression in patients with diabetes (7,8). Protective mechanisms of PPAR $\gamma$  agonists may include favorable changes in plasma lipoprotein profiles and inflammatory markers. For example, RSG can raise HDL-cholesterol levels and lower C-reactive protein levels in patients with type 2 diabetes (9–11). RSG can also lower postprandial triglyceride levels in patients with type 2 diabetes without changes in fasting plasma triglycerides (12). However, favorable lipid effects of RSG may not be as apparent in nondiabetic patients. Even though RSG can lower plasma concentrations of C-reactive protein and IL-6, it also can increase total cholesterol (13), LDL cholesterol, and triglyceride levels (14) in nondiabetic patients. Other endogenous or exogenous factors, such as the overall nutritional status of a patient, may play a role in the effectiveness of PPAR agonists as a broad antiatherogenic agent (15).

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There is evidence that zinc can modulate PPAR signaling (16). The DNA-binding domain (DBD)<sup>11</sup> of PPAR has 2 sets of zinc fingers (17). The specificity and polarity of PPAR-DNA binding seems to be at least in part due to features in the zinc finger domains of PPAR (18). The DNA binding partner of PPAR, retinoid X receptor (RXR), also has a DBD with 2 zinc fingers involved (19). Upon ligand activation, PPAR heterodimerizes with RXR and binds to the PPAR response element (PPRE) within the promoter region of target genes, thereby regulating or transactivating their expression (20). As zinc is an essential constituent of the DBD of both PPAR and RXR, zinc deficiency could impair the function of this transcription factor complex.

Zinc fingers also have been described to mediate protein-lipid interactions. Zinc-containing FYVE domains are specific in recognizing and binding phosphatidylinositol-3-phosphate, a component of cell membrane (21). It is thus very likely that zinc plays a critical role in PPAR signaling and associated regulation of cellular lipid metabolism. Thus, the objective of this study was to explore the role of zinc in the antiatherogenic properties of the PPAR $\gamma$  ligand RSG, with a focus on selected lipid parameters in an atherogenic mouse model. We hypothesize that PPAR signaling and associated lipid metabolism are compromised during zinc deficiency and that adequate dietary zinc may be critical to maintain favorable lipid effects of the antidiabetic medicine RSG.

## Materials and Methods

**Mice and diets.** LDL-receptor (LDL-R)-deficient (LDL-R<sup>-/-</sup>) male mice were obtained from the Jackson Laboratory (stock no. 002207) and housed at Iowa State University. Four mice were housed in each plastic cage with wire mesh floors over wood chip bedding. Cellulose pads were provided to the mice for nesting and a 12-h-light/-dark cycle was maintained. Mice consumed distilled water ad libitum through plastic bottles with plastic stoppers to reduce zinc contamination. All procedures were in compliance with and approved by the ISU Animal Care and Use Committee. LDL-R<sup>-/-</sup> mice were obtained at 5 wk of age, weighed 16.3 g, and were fed a low-fat (LF) diet with either 0.4 mg/kg of zinc (0 Zn, zinc-deficient diet) or 33.1 mg/kg of zinc (30 Zn, zinc-adequate diet). After 21 d of feeding the LF diet, all mice were assigned to a high-fat (HF) diet without changing the original zinc nutritional status. Mice were fed the HF diet for 1 wk. Diets were prepared based on AIN-93 standards (22) but used egg white rather than casein as the protein source to provide a low-zinc diet (23) (Table 1). The diets were of similar energy value: 18.17 kJ/g for the HF and 16.50 kJ/g for the LF diets, respectively. RSG (20 mg·kg<sup>-1</sup>·d<sup>-1</sup>) (24) or the vehicle (0.25% of methylcellulose) were administered for 9 d by oral gavage. RSG treatment was initiated 2 d prior to the start of the HF dietary regimen. Body weights of all mice were measured every 2 d throughout the study. After completion of the study (4 wk), the mice were killed by intraperitoneal phenobarbital injection. Sufficient plasma samples were not available from all animals for glucose analysis, resulting in variations in sample size as outlined.

Food intake was measured over a 3-d period within the first week of the study. Mean food intake did not differ between zinc-deficient and zinc-adequate mice at this time. In other work, we have observed mice fed a zinc depleted diet reduced their food intake compared to the zinc adequate animals in a cyclic pattern with progressively lower food intakes with time. Body weight was unchanged until d 9 but subse-

**TABLE 1** Experimental diets<sup>1</sup>

Ingredient	LF/0 Zn	HF/0 Zn	LF/30 Zn	HF/30 Zn
	<i>g/kg</i>			
Egg white	200.0	200.0	200.0	200.0
DL-Methionine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Corn starch	397.5	397.5	397.5	397.5
Sucrose	100.0	100.0	100.0	100.0
Dyetrose	131.0	51.0	121.0	41.0
Cellulose	35.0	35.0	35.0	35.0
Corn oil	50.0	130.0	50.0	130.0
Safflower oil	20.0	20.0	20.0	20.0
Mineral mix <sup>2</sup>	50.0	50.0	50.0	50.0
Zinc mix <sup>3,4</sup>	0.0	0.0	10.0	10.0
Vitamin mix AIN-93	10.0	10.0	10.0	10.0
Biotin mix <sup>5</sup>	1.0	1.0	1.0	1.0

<sup>1</sup> Diet ingredients were purchased from MP Biomedicals, except for dyetrose, which was purchased from Dyets, and corn starch, sucrose, and corn oil, which were purchased from a local food supply warehouse.

<sup>2</sup> A mineral mix was prepared using elemental compounds in cornstarch to provide a zinc-depleted mixture (22,23).

<sup>3</sup> Zinc carbonate was mixed with dyetrose and added to provide the desired final concentrations in the diets.

<sup>4</sup> Actual zinc concentrations of the zinc-deficient and zinc-adequate diets determined by atomic absorption were  $0.4 \pm 0.1$  and  $33.1 \pm 0.3$  mg/kg, respectively.

<sup>5</sup> Biotin was mixed with dyetrose and added to provide 0.005 g biotin/g egg white protein.

quently increased only in zinc-adequate mice. RSG treatment did not affect body weight in either zinc-adequate or -deficient groups, respectively. Body weights in each group at the end of the feeding study were (mean  $\pm$  SEM): 0 Zn,  $16.43 \pm 1.20$  g; 0 Zn + RSG,  $15.12 \pm 0.52$  g; 30 Zn,  $19.56 \pm 0.26$  g; and 30 Zn + RSG,  $19.29 \pm 0.48$  g.

**Zinc quantification.** We drew blood from exposed hearts using heparinized syringes. Plasma samples were prepared by centrifugation at  $14,000 \times g$ ; 10 min at room temperature. Livers were flash-frozen in liquid nitrogen after excision. Both plasma and liver samples were stored at  $-80^\circ\text{C}$  prior to analysis. Zinc concentration in plasma, liver, and RSG solution was analyzed by inductively coupled plasma MS by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO) (25).

**Plasma cholesterol and lipoprotein-cholesterol distribution.** Plasma total cholesterol content was determined enzymatically using a commercially available kit, Wako Cholesterol E (Wako Chemicals). Plasma cholesterol distribution in different lipoprotein fractions was measured by fast-performance liquid chromatography using a Biologic DuoFlow System (Bio-Rad Laboratories) equipped with a Superose 6HR 10/30 column (Amersham Pharmacia Biotech) (26).

**Plasma fatty acids.** Plasma total lipids were extracted with chloroform (27) followed by methyl esterification of total fatty acids with BF<sub>3</sub>/Methanol (Supelco). Analysis of fatty acids was performed using a GC system, Agilent 6890 GC G2579A system (Agilent) equipped with an OMEGAWAX 250 capillary column (Supelco) and a flame ionization detector. An Agilent 5973 network mass selective detector (Agilent) was used to identify target peaks. We used heptadecanoic acid (17:0) as an internal standard for data analysis.

**Real-time RT-PCR.** Abdominal aorta and liver were excised from the mice, immersed in RNAlater (Qiagen), and stored at  $-80^\circ\text{C}$  until analysis. Total RNA was isolated from abdominal aorta using RNeasy Fibrous Tissue Mini kit (Qiagen) after surrounding adipose and connective tissues were removed and total RNA was isolated from liver using RNeasy Mini kit (Qiagen). cDNA was generated using the Reverse Transcription System (Promega). Gene expression was determined by

<sup>11</sup> Abbreviations used: 0 Zn, zinc-deficient diet; 30 Zn, zinc-adequate diet; DBD, DNA-binding domain; HF, high-fat diet; IDL, intermediate density lipoprotein; LDL-R, LDL-receptor; LDL-R<sup>-/-</sup>, LDL receptor deficient; LF, low-fat diet; LPL, lipoprotein lipase; mRNA, messenger RNA; PPRE, PPAR response element; RAVSMC, rat aortic vascular smooth muscle cell; RSG, rosiglitazone; RXR, retinoid X receptor.

real-time PCR using the ABI Prism 7300 Real Time PCR system (Applied Biosystems) and TaqMan Universal PCR Master mix, No AmpErase UNG (Applied Biosystems). TaqMan gene expression assays were used for mouse fatty acid translocase (FAT/CD36) and lipoprotein lipase (LPL) (Mm00432403\_m1, and Mm 00434764\_m1, Applied Biosystems). Each assay consisted of a specific pair of unlabeled PCR primers and a specific TaqMan MGB probe that was 5' end labeled with a FAM reporter dye and 3' end labeled with a minor groove binder/non-fluorescent quencher (MGBNFQ). Detection of 18S rRNA, or  $\beta$ -actin as endogenous control, utilized predeveloped Taqman assay reagents, i.e. Eukaryotic 18S rRNA Endogenous Control or Mouse ACTB Endogenous Control (Applied Biosystems).

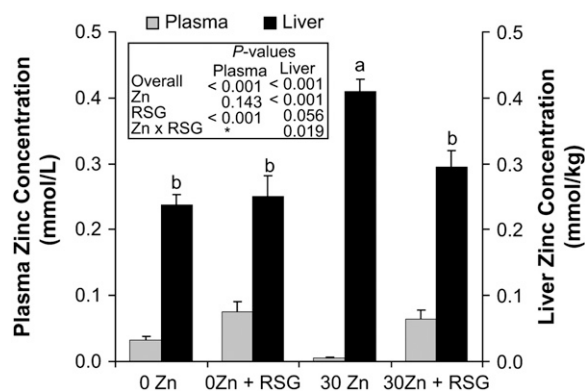
**Plasma glucose.** Plasma glucose concentration was determined using PGO enzymes (Sigma-Aldrich) and *o*-dianisidine dihydrochloride (Sigma-Aldrich) according to the manufacturer's instruction. The amount of glucose in the test sample was measured by the absorbance at 450 nm using a SpectraMax M2 microplate reader (Molecular Devices).

**Transient transactivation and luciferase assay.** Rat aortic vascular smooth muscle cells (RAVSMC) were grown in 6-well plates in DMEM (Invitrogen) containing 10% FBS (Invitrogen). Media were changed to DMEM containing 2% FBS without antibiotics, in which the cells were treated with 600 nmol/L of the zinc chelator diethylenetriaminepentaacetic acid (DTPA) (Sigma-Aldrich) with or without 600 nmol/L ZnSO<sub>4</sub> for 24 h. Subsequently, 400 ng DNA of the acyl-CoA oxidase PPRE-Tk-luciferase reporter construct and 200 ng of the full-length PPAR $\gamma$ 1 expression vector were cotransfected using Lipofectamine 2000 (Invitrogen) and OPTI-MEM I (Invitrogen) (28). After transfection for 6–8 h, cells were stimulated with 10  $\mu$ mol/L RSG for 24 h. Luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. Transfection efficiency was adjusted by normalizing firefly luciferase activities to the renilla luciferase activities generated by cotransfection with 10 ng pGL4.74 [hRluc/TK] (Promega).

**Statistical analysis.** Data were expressed as means  $\pm$  SEM and analyzed using SPSS 12.0 and JMP 7.0 (SAS). Zinc and RSG were used as explanatory variables in 2-way ANOVA models. Nonsignificant interactions were removed from the models. Post hoc comparisons were conducted using the least significance difference method only when there were significant interactions in the 2-way model. One-way ANOVA with post hoc comparisons using the least significance difference method was used to analyze the in vitro data.  $P < 0.05$  was considered significant. Actual  $P$ -values were reported when  $< 0.1$ .

## Results

Plasma zinc concentrations did not differ between the LDL-R<sup>-/-</sup> mice fed the 0 Zn diet and the 30 Zn diet regardless of RSG treatment (Fig. 1). Treatment with RSG increased plasma zinc concentrations in both dietary groups ( $P < 0.001$ ; Fig. 1). Liver zinc concentration was lower in untreated mice fed the 0 Zn diet than mice fed the 30 Zn diet ( $P < 0.001$ ; Fig. 1). In mice fed the



**FIGURE 1** Effects of dietary zinc status and RSG on plasma and liver zinc concentrations in LDL-R<sup>-/-</sup> mice. Values are means  $\pm$  SEM,  $n = 9$ –15. \*Zn  $\times$  RSG interaction was not significant ( $P > 0.05$ ).

30 Zn diet only, RSG treatment reduced liver zinc concentration ( $P = 0.002$ ; Fig. 1).

RSG treatment contributed to a predictable biological outcome by affecting plasma insulin and adiponectin concentrations. Administration of RSG resulted in a 36% decrease in plasma insulin concentrations and a 2-fold increase in adiponectin concentrations (Table 2).

**Zinc deficiency elevates plasma total cholesterol in LDL-R<sup>-/-</sup> mice.** The 0 Zn diet resulted in higher concentrations of plasma total cholesterol in LDL-R<sup>-/-</sup> mice compared with 30 Zn diet (Fig. 2). Treatment with RSG increased the concentration of plasma total cholesterol (Fig. 2).

**Zinc deficiency increases non-HDL cholesterol distribution in LDL-R<sup>-/-</sup> mice.** Consistent with the data on plasma total cholesterol, zinc deficiency increased concentrations of plasma cholesterol contained in the non-HDL fraction [VLDL, intermediate density lipoprotein (IDL), LDL] as compared with zinc-adequate mice (area under the curves;  $P < 0.001$ ; Fig. 3). In contrast, levels of HDL cholesterol were similar in all mice independent of the dietary zinc intake (Fig. 3). RSG treatment did not affect lipoprotein-cholesterol profile under either zinc-adequate or zinc-deficient conditions (Fig. 3).

**Zinc deficiency elevates plasma fatty acid concentrations in LDL-R<sup>-/-</sup> mice.** The major plasma fatty acids are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and arachidonic acid (20:4), which comprise  $\sim 90\%$  of total plasma fatty acids (29). Plasma total fatty acids in LDL-R<sup>-/-</sup> mice tended to increase with zinc deficiency ( $P = 0.080$ ; Table 3).

**TABLE 2** Effects of dietary zinc status and RSG on plasma glucose, insulin, and adiponectin concentrations in LDL-R<sup>-/-</sup> mice<sup>1</sup>

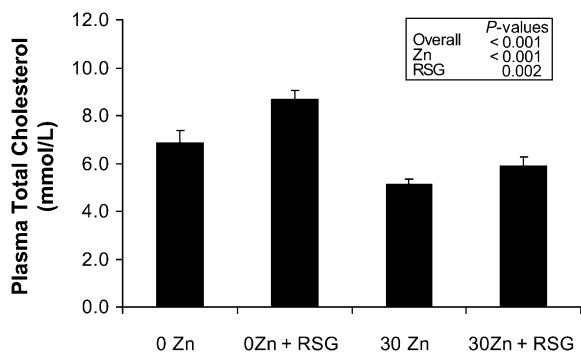
	0 Zn	0 Zn + RSG	30 Zn	30 Zn + RSG	$P$ -values <sup>2</sup>		
					Overall	Zn	RSG
Glucose, <sup>3</sup> mmol/L	15.6 $\pm$ 1.6	13.1 $\pm$ 0.9	17.2 $\pm$ 0.9	16.0 $\pm$ 1.1	0.0076	0.0319	0.1455
Insulin, <sup>4</sup> pmol/L	118.5 $\pm$ 18.1	78.5 $\pm$ 16.7	176.3 $\pm$ 18.1	110.6 $\pm$ 16.7	0.0024	0.0176	0.0055
Adiponectin, <sup>4</sup> nmol/L	511.2 $\pm$ 121.5	1495.5 $\pm$ 112.2	328.2 $\pm$ 121.5	1056.6 $\pm$ 121.5	0.0001	0.0149	0.0001

<sup>1</sup> Values are means  $\pm$  SEM.

<sup>2</sup>  $P$ -values from 2-way ANOVA. Zn  $\times$  RSG interactions were not significant,  $P > 0.05$ .

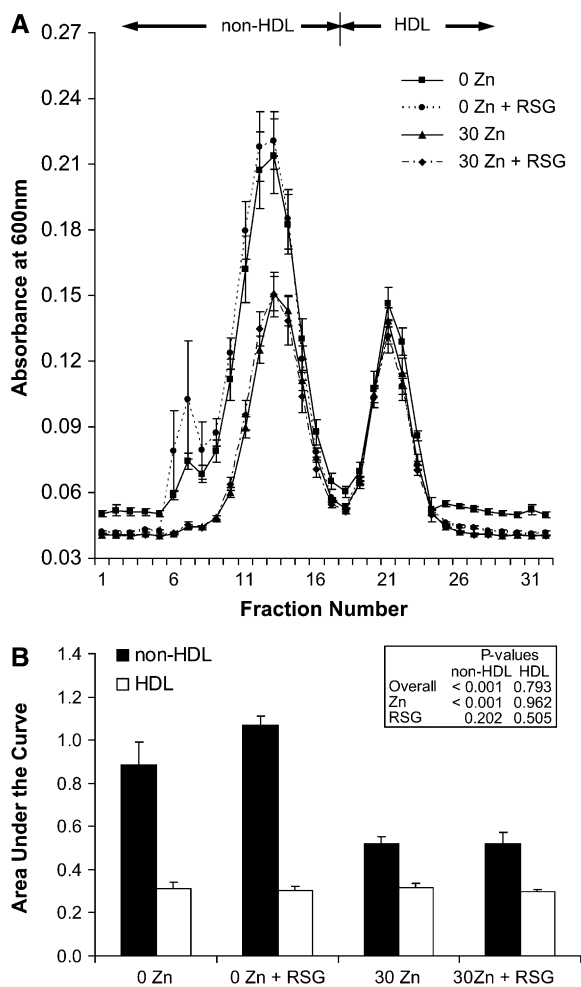
<sup>3</sup>  $n = 4$ –14.

<sup>4</sup>  $n = 6$ –7.



**FIGURE 2** Effects of RSG and dietary zinc status on plasma total cholesterol concentration in LDL-R<sup>-/-</sup> mice. Values are means ± SEM, n = 10–15. Zn × RSG interaction was not significant (P > 0.05).

Detailed analysis of the fatty acid profile revealed that the patterns of fatty acid changes due to RSG treatment (except for 20:4) were similar and that elevated levels of 18:0 and 18:1 in zinc-deficient LDL-R<sup>-/-</sup> mice were mostly responsible for the increased total fatty acid levels (Table 3).



**FIGURE 3** Effects of dietary zinc status and RSG on cholesterol distribution in different lipoprotein fractions in LDL-R<sup>-/-</sup> mice. (A) Lipoprotein-cholesterol distribution. An equal amount (50 μL) of individual plasma samples was applied to the fast-performance liquid chromatography column. The non-HDL includes VLDL, IDL, and LDL. (B) Area under the curve. Values are means ± SEM, n = 4. Zn × RSG interactions were not significant (P > 0.05).

The effects of RSG on expression of genes associated with lipid uptake and metabolism in LDL-R<sup>-/-</sup> mice are regulated by zinc status. LPL is an enzyme that hydrolyses triglyceride-rich lipoproteins and CD36 mediates cellular uptake of FFA. Zinc status did not affect the baseline LPL and CD36 messenger RNA (mRNA) expression (Fig. 4). Treatment with RSG upregulated LPL mRNA expression in livers (P = 0.014; Fig. 4).

The effects of RSG on CD36 gene expression in abdominal aortas were also regulated by zinc status. Specifically, CD36 mRNA levels increased 1.75-fold in zinc-deficient LDL-R<sup>-/-</sup> mice that received RSG treatment (P = 0.013; Fig. 4). These effects were not observed in zinc-adequate mice (Fig. 4).

**Adequate zinc is required for functional activity of PPARγ.** PPARγ transactivation activity in PPARγ and PPARE cotransfected RAVSMC was induced by RSG in zinc-adequate cells (P < 0.001; Fig. 5). Zinc deficiency caused by DTPA inhibited PPARγ transactivation activity induced by RSG (P = 0.017; Fig. 5), which could be reversed by zinc supplementation (P = 0.045; Fig. 5).

## Discussion

This study was designed to investigate the interaction of a modest zinc depletion and dietary fat intake on the response to RSG in mice lacking LDL-R. To our knowledge, this is the first study to investigate the effect of RSG and dietary zinc depletion in this strain of mice. Based on our preliminary study with LDL-R normal mice and the short duration of the study, we did not expect plasma zinc concentrations to be significantly affected. And, in fact, plasma zinc concentrations did not differ between mice fed the 0 zinc and 30 Zn diets regardless of RSG treatment. However, within the dietary zinc groups (0 zinc or 30 zinc), RSG treatment increased plasma zinc concentrations. We verified that the RSG solution was zinc-free; therefore, this repartitioning of plasma zinc by RSG appears to be a specific effect of the drug. Liver zinc concentration is considered to be more responsive to dietary zinc intake than plasma zinc (30) and indeed we observed lower liver zinc concentrations in mice fed the low-zinc diet than the adequate-zinc diet. However, with RSG treatment, liver zinc was reduced only in the mice fed the 30 Zn diet. The observation that RSG increased plasma zinc in both dietary groups suggests that other body zinc stores besides liver may be mobilized by RSG and this observation is worthy of further study.

Zinc is critical for normal function of numerous proteins. Thus, a change in cellular zinc status can affect multiple cellular events. Because PPAR plays a role in lipid transport and metabolism (31), lack of zinc appears to result in dysfunctional PPAR signaling with a subsequent detrimental lipid metabolism. PPARγ activation upregulates the expression of adiponectin, a PPARγ target gene (32), which promotes insulin sensitivity and downregulates inflammatory cytokines and thus insulin resistance (15,33). Most importantly, PPARγ activates numerous genes involved in lipid storage and lipogenesis (15) and in particular in the cellular assimilation of lipids via anabolic pathways (34). Whether or not the overall antiatherogenic properties of PPARγ agonists are due to favorable lipid changes or antiinflammatory properties is not clear. However, protection against cardiovascular complications by PPARγ agonists is well accepted. For example, RSG strongly inhibited the development of atherosclerosis in LDL-R<sup>-/-</sup> mice (24).

**TABLE 3** Effects of dietary zinc status and RSG on plasma total and individual fatty acid concentrations in LDL-R<sup>-/-</sup> mice<sup>1</sup>

Fatty acid	0 Zn	0 Zn + RSG	30 Zn	30 Zn + RSG	P-values <sup>2</sup>		
					Overall	Zn	RSG
	<i>mmol/L</i>						
Total	9.39 ± 0.61	11.15 ± 0.56	8.67 ± 0.83	9.25 ± 0.78	0.062	0.080	0.150
16:0	1.85 ± 0.16	2.19 ± 0.13	1.69 ± 0.13	1.82 ± 0.13	0.039	0.059	0.115
18:0	1.23 ± 0.17	1.36 ± 0.14	0.95 ± 0.13	0.90 ± 0.14	0.045	0.015	0.837
18:1	1.52 ± 0.14	1.82 ± 0.12	1.10 ± 0.11	1.35 ± 0.12	<0.001	<0.001	0.031
18:2	3.40 ± 0.33	4.22 ± 0.28	3.38 ± 0.26	3.55 ± 0.28	0.128	0.232	0.130
20:4	1.40 ± 0.16	1.56 ± 0.14	1.55 ± 0.13	1.63 ± 0.14	0.557	0.453	0.393

<sup>1</sup> Values are means ± SEM, *n* = 5–8.

<sup>2</sup> P-values from 2-way ANOVA. Zn × RSG interactions were not significant, *P* > 0.05.

The role of zinc deficiency in atherosclerosis is not well defined; however, epidemiological studies suggest that in some population groups, low serum concentrations of zinc are associated with coronary artery disease (35). Although controversy still exists about the effect of zinc on human lipoprotein metabolism, some studies confirmed the lipid-lowering effects of zinc in humans. Oral zinc supplementation decreased total and LDL cholesterol, whereas HDL cholesterol increased in both normal and diabetic humans (36,37). Other studies, however, found that zinc supplementation had little effect on lipoprotein profiles (38) or decreased HDL cholesterol (39,40).

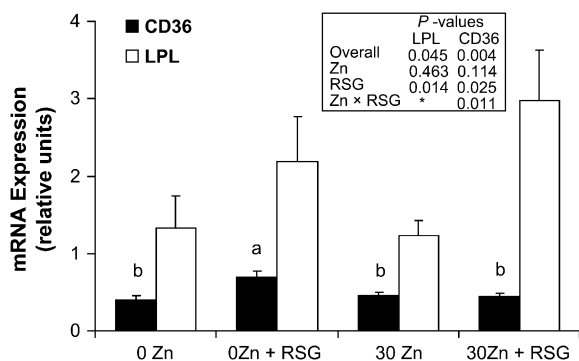
In addition to its antioxidant and antiinflammatory properties (41–44), we have demonstrated previously that the expression of both PPAR $\gamma$  (16) and PPAR $\alpha$  (our unpublished data) at both the mRNA and protein levels was significantly reduced during cellular zinc deficiency and that this effect was reversible by zinc supplementation. In this same cell model, PPAR $\gamma$ - and PPAR $\alpha$ -specific agonists induced PPAR DNA-binding activity, which was compromised during zinc deficiency (45). Using a transactivation assay, we demonstrated in the current study that zinc deficiency inhibited RSG-induced PPAR $\gamma$  activity and that this effect can be reversed by zinc supplementation.

In the present *in vivo* study, we also provide evidence that PPAR $\gamma$ -regulated gene expression and associated lipid metabolism are compromised during zinc deficiency and that adequate dietary zinc may be critical to maintain favorable lipid effects of RSG. Furthermore, RSG treatment decreased inducible nitric

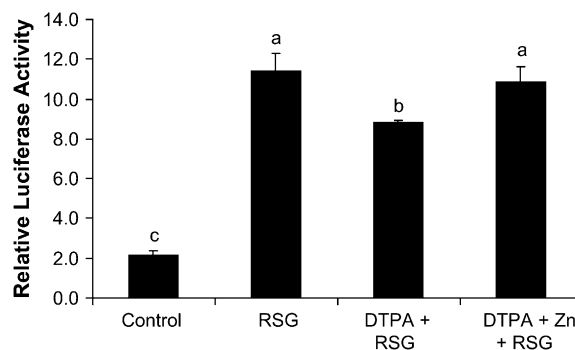
oxide synthase (iNOS) mRNA expression in abdominal aortas and circulating IL-12 levels only in zinc-adequate mice but not in zinc-deficient mice (data not shown), suggesting that the antiinflammatory properties of RSG can be compromised during zinc deficiency. Treatment with RSG tended to increase plasma total cholesterol more in zinc-deficient mice. Such lipid change is atherogenic and suggests that any possible favorable lipid profile induced by RSG treatment may be compromised during zinc deficiency. Furthermore, zinc deficiency alone caused a shift of lipoprotein-cholesterol distribution to the non-HDL (VLDL, IDL, and LDL) fraction. This is consistent with our previous findings that zinc deficiency can increase plasma lipids and atherosclerotic markers in LDL-R<sup>-/-</sup> mice (46).

Although many studies suggest that treatment with PPAR $\gamma$  agonists such as RSG stabilizes or improves plasma lipid parameters, especially in diabetic patients (47–49), other studies reported significantly increased triglycerides following treatment with RSG (14,50,51). In the LDL-R<sup>-/-</sup> mouse model, we observed an elevation of total plasma fatty acids in zinc-deficient mice treated with RSG. All major plasma fatty acids appeared to be elevated in the zinc-deficient group receiving RSG. There is clear evidence that hypertriglyceridemia is an independent risk factor of cardiovascular diseases such as atherosclerosis (52,53). Furthermore, triglyceride-rich lipoproteins and FFA are often elevated in patients with type 2 diabetes and are thus a major risk factor (54,55).

Our data suggest that expression of the LPL gene, which is a PPAR $\gamma$  target gene (56) and is also critical in the clearance of



**FIGURE 4** Effects of RSG and dietary zinc status on LPL and CD36 gene expression in LDL-R<sup>-/-</sup> mice. The vertical axis represents relative units, calculated as the ratio of the copy number of the target gene over the copy number of the endogenous control (18S rRNA and  $\beta$ -actin, respectively). Values are means ± SEM, *n* = 10–15 for LPL and 7–9 for CD36 mRNA expression, respectively. \*Zn × RSG interaction was not significant (*P* > 0.05).



**FIGURE 5** Effect of zinc status on RSG-induced PPAR $\gamma$  transactivation in PPAR $\gamma$  and PPRE cotransfected RAVSMC. PPAR $\gamma$  transactivation activity was measured as relative luciferase activity (firefly luciferase activity: renilla luciferase activity). Means without a common letter differ, *P* < 0.05. Values are means ± SEM, *n* = 3. The results represent the results of 3 repeated experiments.

triglyceride-rich lipoproteins, was upregulated in zinc-adequate mice upon treatment with RSG. Other researchers observed similar results in brown adipose tissue of rodents treated with this PPAR $\gamma$  agonist (48). In contrast, mRNA expression of LPL was minimally upregulated in zinc-deficient mice as a result of RSG treatment, which may be due to compromised PPAR $\gamma$  function. Because LPL is critical in clearance of triglyceride-rich lipoproteins and is able to limit inflammation by generating endogenous PPAR $\alpha$  ligands (thus mediating PPAR $\alpha$  activation) (57), dysfunction of this gene due to zinc deficiency could further contribute to lipid risk factors of atherosclerosis.

Scavenger receptors like CD36 are important in the early pathology of atherosclerosis, which includes macrophage uptake of modified LDL and foam cell formation (58). In fact, the absence of CD36 in ApoE-deficient mice resulted in a marked decrease in total lesion area (58). There is also evidence that increased CD36 is caused by defective insulin signaling and that administration of PPAR $\gamma$  agonists can decrease CD36 protein (59). In our study, CD36 gene expression in abdominal aorta was significantly upregulated by RSG only in zinc-deficient mice, suggesting accelerated uptake of lipids and especially prooxidative and proinflammatory fatty acids. In contrast, in another study, RSG upregulated aortic CD36 mRNA in mice consuming a high-cholesterol diet (24). There is evidence using human macrophages that CD36 upregulation by darglitazone, another PPAR $\gamma$  ligand, is modified by the presence or absence of physiological concentrations of albumin-bound oleic or linoleic acid (60). In this study, RSG treatment resulted in elevated concentrations of plasma total cholesterol and total fatty acids in zinc-deficient mice, which could increase cellular oxidative stress. This may be sufficient to activate the redox-sensitive transcription factor nuclear erythroid-2 related factor 2 (61), which is another important transcription factor involved in the induction of CD36 besides PPAR $\gamma$  (62). Indeed, oxidative stress has been found to increase the expression of CD36 in macrophages from atherosclerotic mice (63). Therefore, the upregulation of CD36 by RSG in zinc-deficient mice could be due in part to the activation of nuclear factor erythroid-2 related factor 2 caused by increased oxidative stress. Our data suggest that treatment with RSG during a nutritional state of zinc deficiency may increase, rather than decrease, hyperlipidemic risk factors.

There are some unexpected results in our study. For example, the similar effects of RSG on adiponectin levels in mice fed either zinc-deficient or zinc-adequate diets suggest that adiponectin gene expression may be only partially regulated by a PPAR $\gamma$ -dependent pathway and that RSG may also regulate the expression of adiponectin via PPAR $\gamma$ -independent pathways (64). Therefore, it is likely that some PPAR $\gamma$ -independent pathway that is not zinc dependent contributed to the observed effects of RSG treatment on adiponectin levels.

In summary, we are providing *in vivo* evidence that zinc deficiency interacts with RSG treatment to induce selected proatherogenic lipid profiles in LDL-R<sup>-/-</sup> mice. Our data also illustrate that adequate dietary zinc is critical for preventing or minimizing some possible side effects of antidiabetic PPAR $\gamma$  agonists. For example, CD36 gene expression in abdominal aorta was significantly upregulated by RSG only in zinc-deficient mice. Even though not significant, treatment with RSG tended to increase plasma total cholesterol and fatty acids more when mice were zinc deficient. Because dietary zinc intake of certain population groups is still below intake recommendations (65), these data emphasize the importance of adequate dietary zinc in humans during treatment phases associated with diabetes and other cardiovascular risk factors.

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