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RESEARCH ARTICLES

Dietary zinc restriction in rats alters antioxidant status and increases plasma F_2 isoprostanes☆

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Abstract

Approximately 12% of Americans do not consume the estimated average requirement for zinc and could be at risk for zinc deficiency. Since zinc has proposed antioxidant function, inadequate zinc consumption may lead to an enhanced susceptibility to oxidative stress through several mechanisms, including altered antioxidant defenses. In this study, we hypothesized that dietary zinc restriction would result in lower antioxidant status and increased oxidative damage. We fed weanling Sprague–Dawley rats ($n=12$ per group) a zinc-adequate (50 mg/kg of zinc) diet, a zinc-deficient (<0.05 mg/kg of zinc) diet or a pair-fed diet for 3 weeks and then assessed their antioxidant status and oxidative stress parameters. Rats were zinc deficient as indicated by a significant ($P<.05$) reduction in body weight (49%) and 19% lower ($P<.05$) hepatic zinc (20.6±2.1 mg/kg) as compared with zinc-adequate rats (24.6±2.2 mg/kg). Zinc deficiency resulted in elevated ($P<.05$) plasma F_2 isoprostanes. Zinc deficiency-mediated oxidative stress was accompanied by a 20% decrease ($P<.05$) in the ferritin-reducing ability of plasma assay and a 50% reduction in plasma uric acid ($P<.05$). No significant change in plasma ascorbic acid or in plasma α-tocopherol and γ-tocopherol was observed. However, hepatic α-tocopherol and γ-tocopherol concentrations were decreased by 38% and 27% ($P<.05$), respectively, as compared with those in zinc-adequate rats. Hepatic α-tocopherol transfer protein levels were unaltered ($P>.05$) by zinc deficiency, but cytochrome *P*450 (CYP) 4F2 protein levels were elevated ($P<.05$) as compared with those in zinc-adequate rats. Collectively, zinc deficiency increased oxidative stress, which may be partially explained by increased CYP activity and reductions in hepatic α-tocopherol and γ-tocopherol and in plasma uric acid.

Keywords: Zinc deficiency; Oxidative stress; Isoprostanes; Antioxidants; Vitamin E; Vitamin C; FRAP; CYP4F2; TTP

1. Introduction

Zinc is the most abundant trace intracellular element required for a number of cellular processes, including cell proliferation, reproduction, immune function and defense against free radicals [1,2]. It is a component of more than 1000 zinc-associated transcription factors, including DNA-binding proteins with zinc fingers, and is required in more than 300 zinc-containing metalloenzymes.

A significant proportion of North Americans do not ingest adequate zinc [3]. Based on the 2000–2001 NHANES data, approximately 12% of Americans do not consume the estimated average requirement for zinc and could be at risk for zinc deficiency [4]. Populations particularly at risk are those who consume little meat and/or consume high phytate-containing food [5]. Zinc deficiency increases the risk for several chronic disease states, such as cancer [6], and this risk may be associated with an increased vulnerability to oxidative stress [7]. Indeed, increasing evidence suggest that zinc plays an important role as an antioxidant and protects cellular components from oxidation. However, the specific mechanisms by which zinc functions as an antioxidant remain unclear. Several laboratories, including our own, have demonstrated increased oxidative stress with low cellular zinc [8–10]. Cell cultures grown in zinc-deficient

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media produce increased levels of oxidants [10]. Oxidative stress biomarkers, specifically oxidized protein and DNA damage, are increased in zinc-deficient rats [3,5,7,11,12]. Additionally, increased free radical production or increased oxidative damage occurs in response to zinc deficiency in vitro and in vivo. For example, increases in hyperoxic lung damage, carbon tetrachloride toxicity and increased lipid peroxidation were observed in zinc-deficient rats as compared with their zinc-adequate and pair-fed control counterparts [5,7,13,14]. Thus, these studies clearly illustrated that zinc deficiency increased the vulnerability of animals to oxidative insults.

The in vivo interaction between zinc and other antioxidant nutrients and the mechanisms by which zinc deficiency alters sensitivity to oxidative stress have not been well established. We hypothesized that zinc deficiency in rats would increase oxidative stress and compromise their ability to maintain an adequate antioxidant status. Therefore, we examined antioxidant capacity, oxidative stress and interactions with other antioxidant nutrients, such as vitamins E and C, using an acute zinc deficiency model with weanling rats.

2. Materials and methods

2.1. Materials

Perchloric acid and high-performance liquid chromatography (HPLC)-grade methanol were obtained from Fisher (Fair Lawn, NJ, USA). The following were obtained from Sigma-Aldrich (St. Louis, MO, USA): ascorbic acid; butylated hydroxytoluene; DTPA (diethylenetriamine pentaacetic acid); $FeCl_3$; phosphate-buffered saline; potassium hydroxide; potassium phosphate trihydrate; TPTZ [2, 4, 6-tri(2-pyridil)-*s*-triazine]; and trolox. The chromatography pairing reagent Q12 (1-dodecyltriethyl-ammonium phosphate) was purchased from Regis (Morton Grove, IL, USA). α-Tocopherol and γ-tocopherol standards were kind gifts from James Clark (Cognis Nutrition and Health, La-Grange, IL, USA). Ultrapure nitric acid was obtained from VWR (West Chester, VA, USA).

2.2. Animals, test diets and study design

The animal protocol was approved by the Oregon State University (Corvallis, OR, USA) Institutional Laboratory Animal Care and Use Committee. Three-week-old Sprague–Dawley rats were purchased, housed in stainless steel suspended cages and acclimated for 1 week to the temperature- and humidity-controlled environment with a light period between 0600 h and 1800 h. Rats ($n=36$) with an initial body weight of 77.7±3.4 g (mean±S.D.) were randomly assigned to one of three treatments: zinc-adequate (50 mg/kg of zinc) diet; zinc-deficient (<0.5 mg/kg of zinc) diet; or pair feeding in which the rats were fed with the zinc-adequate diet but at quantities ingested by the zinc-deficient group. Animals were fed diets for 21 days based on a modified AIN-93G rodent diet [15], with zinc provided as zinc carbonate (Dyets, Bethlehem, PA, USA).

The zinc-adequate and pair-fed diets contained 50 mg/kg of zinc, whereas the zinc-deficient diet contained <0.5 mg/kg of zinc (Table 1). Vitamin E content was confirmed by HPLC, and the three test diets contained both α-tocopherol (44.5±1.9 mg/kg) and γ-tocopherol (10.4±0.1 mg/kg). The dietary intakes and body weights of all rats were monitored and recorded daily. Animals were allowed free access to their diet except during the fasting period (10–12 h) before they were to be killed.

2.3. Sample handling

At the termination of the experiment, rats were sacrificed following anesthesia with isoflurane (1–5%; Henry Schein, Melville, NY, USA); their blood samples were collected by cardiac puncture into vacutainer blood tubes containing sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) and placed on ice for less than 30 min. Plasma was obtained by centrifugation (500×*g*; 15 min; 4°C; model TJ-6, Beckman, Palo Alto, CA, USA), aliquotted into cryovials, snap frozen in liquid nitrogen and stored at −80°C until analyzed. A portion of plasma was also acidified (1:1) with 10% perchloric acid containing 1 mmol/L of DTPA. This sample was centrifuged (15,000×*g*; 5 min; 4 °C; Centrifuge 5415R, Eppendorf, Hamburg, Germany); the supernatant fraction was removed, snap frozen and stored at −80°C for future analysis of ascorbic acid and uric acid. Tissues, immediately upon harvesting, were rinsed with saline, blotted dry, snap frozen in liquid nitrogen and stored at −80°C until analyzed.

2.4. Biochemical analyses

2.4.1. Zinc

Zinc concentrations were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES; Teledyne Leeman Labs, Hudson, NH, USA) with minor modifications to a previously described method [16]. Briefly, approximately 100 mg of each sample was digested in 1 ml of 70% ultrapure nitric acid overnight. Following

Table 1
Diet composition

	Zinc-adequate diet	Zinc-deficient diet
Egg whites	200	200
Cornstarch	381.364	373.986
Dyetrose (maltodextrin)	127.122	124.5
Dextrose	100	120
Soybean oil	70	70
t-Butylhydroquinone	0.014	0.014
Cellulose	50	50
Salt mix no. 215264	35	35
Vitamin mix no. 310025	10	10
Lab premix no. 410750	10	10
Biotin	4.0	4.0
Choline bitartrate	2.5	2.5
Zinc carbonate premix	10	0

Data are expressed as grams per kilogram. The salt mix was prepared without zinc. The biotin/sucrose premix measured 1 mg of biotin/g. The zinc carbonate premix measured 5 mg of zinc/g.

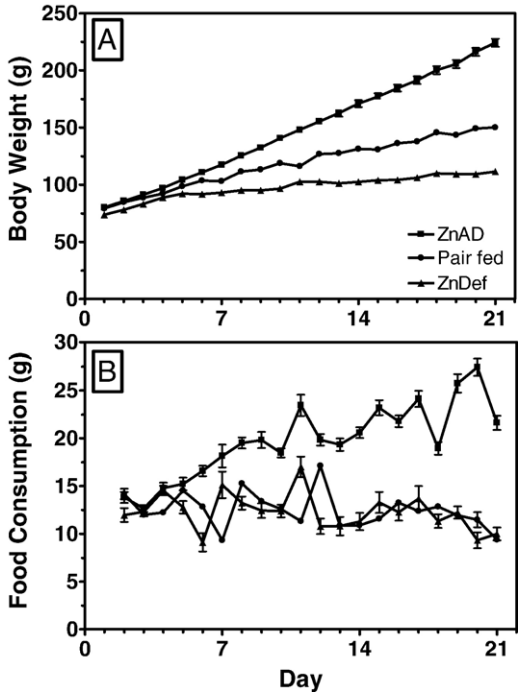

Fig. 1. Body weights (A) and dietary intakes (B) of the rats fed with either a zinc-adequate diet or a zinc-deficient diet throughout the 21-day intervention. Four-week-old Sprague–Dawley rats ($n=12$ per group) were fed with a zinc-adequate (50 mg/kg of zinc) diet or a zinc-deficient (<0.5 mg/kg of zinc) diet. Additionally, a pair-fed group received a zinc-adequate diet but at the level of consumption of the rats fed with the zinc-deficient diet. For clarity, data are presented as mean±S.E. Body weights were significantly reduced ($P<.0001$) in the rats fed with the zinc-deficient diet as compared with those fed with the zinc-adequate diet and those that received the pair-fed diet; the pair-fed rats had body weights that were 26% greater than those of the zinc-deficient rats. Dietary intakes were significantly less ($P<.0001$) in the rats fed with the zinc-deficient diet as compared with the rats fed with the zinc-adequate diet and were matched in those that were pair fed. Abbreviations: ZnDef, zinc deficient; ZnAD, zinc adequate.

digestion, samples were diluted with deionized water in a 7% acid solution and then analyzed by ICP-OES.

2.4.2. Isoprostanes

Total (free and esterified) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), F_2-isoprostane (IsoP) species [8-iso-15(R)-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ and the sum of various IsoPs with the appropriate mass/charge ratio and fragmentation characteristics] and arachidonic acid were measured in plasma as previously described [17]. The sum of IsoPs ($\sum F_2$ IsoP) was determined by integrating the entire region of the chromatogram from 15.8 to 23.7 min during the HPLC separation. Briefly, plasma was subjected to alkaline hydrolysis, acidified, extracted with ethyl acetate–hexane and dried under N_2 gas. The concentrated extract was separated by HPLC (Shimadzu HPLC system; two LC-10ADvp pumps, a DGU-14A degasser, an SIL-HTC autosampler/system controller and a CTO-10Avp column oven). The PGF analytes were detected using multiple reaction monitoring on an Applied Biosystems/MDS Sciex API 3000 triple quadrupole mass spectrometer. Deuterated internal standards of IsoPs (d_4-8-iso-$PGF_{2\alpha}$ and d_4-$PGF_{2\alpha}$) and arachidonic acid (d_8-arachidonic acid) were used to ensure reliable quantitation.

2.4.3. Ferritin-reducing ability of plasma

To assess plasma antioxidant capacity, we measured the ferritin-reducing ability of plasma (FRAP) as previously described [18]. Briefly, diluted plasma samples (1:4) were mixed on a 96-well plate with 300 μl of freshly prepared FRAP reagent [25 ml of sodium acetate buffer (300 mmol/L), 2.5 ml of TPTZ (10 mmol/L) and 2.5 ml of $FeCl_3$ (20 mmol/L)]. Samples were incubated for 15 min at 37°C before they were read at 550 nm on a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). FRAP values were calculated using trolox as a standard.

2.4.4. Vitamin E and CEHC

Plasma and tissue α-tocopherol and γ-tocopherol concentrations were extracted and measured by HPLC electrochemical detection as previously described [19,20]. Concentrations of the α-tocopherol and γ-tocopherol standards were determined spectrophotometrically using 292 nm^{EtOH}=3270 $M^{-1}cm^{-1}$ for α-tocopherol and 298 nm^{EtOH}=3810 $M^{-1}cm^{-1}$ for γ-tocopherol [19]. Hepatic α-CEHC (carboxyethyl-hydroxychroman) and γ-CEHC, the respective physiologic metabolites of α-tocopherol and γ-tocopherol, were measured by LC/MS as previously described [21–23].

2.4.5. Ascorbic acid and uric acid

Plasma ascorbic acid and uric acid were measured by HPLC electrochemical detection as previously described [24,25]. Ascorbic acid standard was prepared fresh and verified spectrophotometrically using 265 nm=14,500 $M^{-1}cm^{-1}$ [24]. Prepared uric acid standard was purchased from Pointe Scientific (Lincoln Park, MI, USA).

Table 2
Zinc status of the Sprague–Dawley rats fed with a zinc-adequate diet, a zinc-deficient diet and a pair-fed diet for 21 days

Parameter	Zinc-adequate diet ($n=12$)	Zinc-deficient diet ($n=12$)	Pair-fed diet ($n=12$)	P
Dietary zinc (mg/kg)	50	<0.5	50	Not determined
Plasma zinc (μg/ml)	8.6±4.1[a]	9.5±3.0[a]	7.2±3.4[a]	.28
Hepatic zinc (mg/kg)	24.6±2.2[a]	20.6±2.1[b]	28.8±2.3[c]	<.0001

Statistical analysis was performed using one-way ANOVA. The P values shown in the table are the main effects of the analysis. Values (mean±S.D.) across a row with different superscript letters are significantly different ($P<.05$) as determined by Tukey's post hoc analysis. At the completion of the investigation, the rats fed with the zinc-deficient diet had unchanged plasma zinc concentrations but had 17% lower hepatic zinc concentrations as compared with the zinc-adequate rats.

2.5. Western blotting: α-tocopherol transfer protein and cytochrome P450

Frozen portions of liver were placed in lysis buffer, homogenized and centrifuged (15,000 rpm; 5 min), and the supernatant was collected. Protein concentrations were determined using the Lowry assay [26]. Proteins (10–20 μg per lane) were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) on a 4–12% bis–Tris gel (Novex, San Diego, CA, USA) and transferred to the nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA). Equal protein loading was confirmed with Ponceau S staining and β-actin level determination. The membrane was blocked for 1 h with 5% nonfat milk, followed by overnight

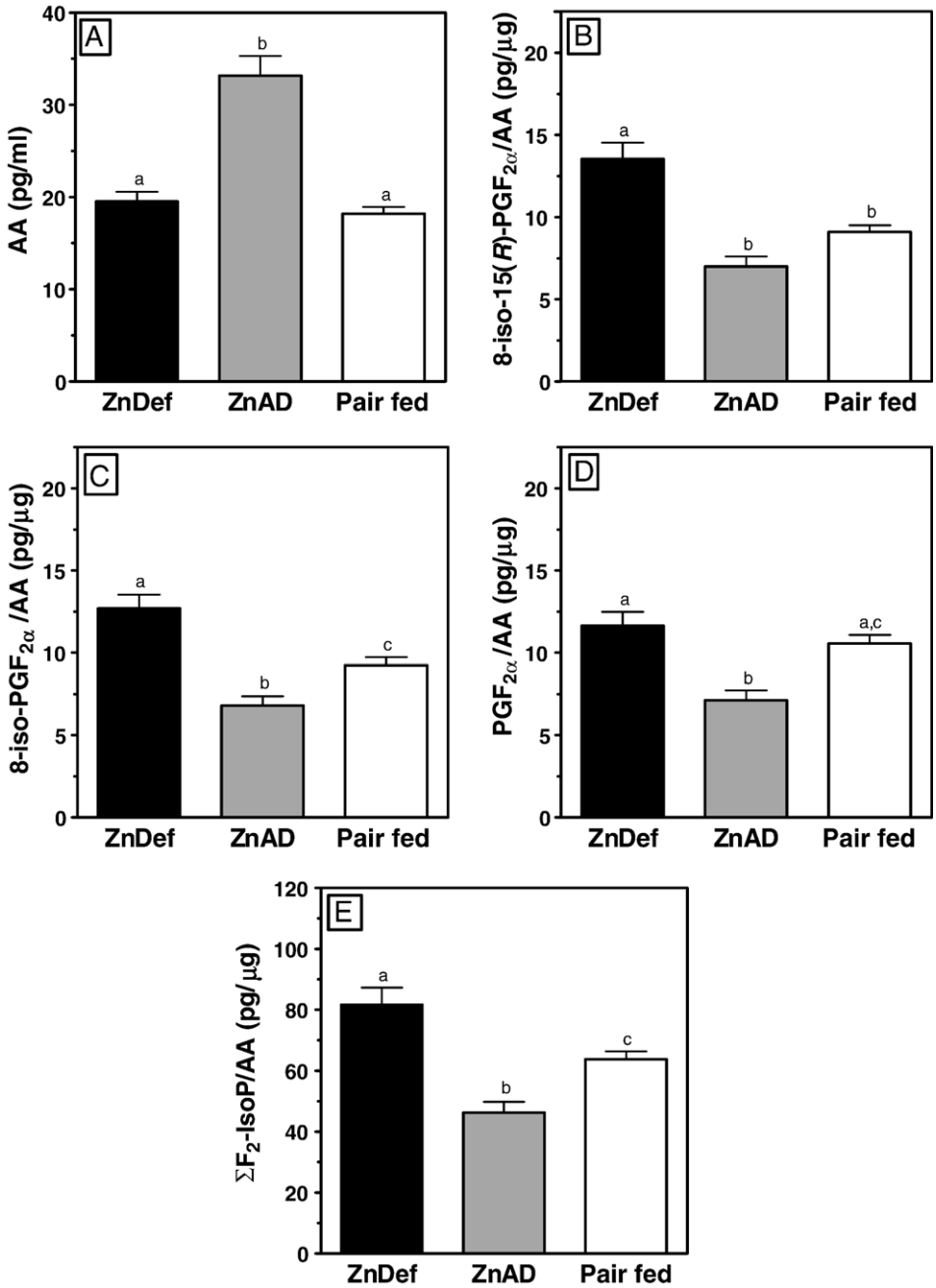

Fig. 2. (A) AA levels; (B) 8-iso-15R-PGF2α normalized to AA; (C) 8-iso-PGF2α normalized to AA; (D) PGF2α normalized to AA; (E) Total isoprostanes normalized to AA. Zinc deficiency decreased plasma arachidonic acid and increased plasma F_2 IsoP. Rats ($n=12$ per group) were fed with a zinc-adequate (50 mg/kg of zinc) diet, a zinc-deficient (<0.5 mg/kg of zinc) diet or a pair-fed (50 mg/kg of zinc) diet for 21 days. Groups having a different notation were significantly different ($P<.05$) as determined by one-way ANOVA with Tukey's post hoc analysis. Arachidonic acid and F_2-IsoP species were determined by LC/MS/MS analysis. Zinc-deficient and pair-fed rats had significantly lower plasma arachidonic acid concentrations; therefore, arachidonic acid was used to normalize plasma F_2 IsoP. The zinc-deficient rats had significantly higher 8-iso-15R-$PGF_{2\alpha}$, 8-iso$PGF_{2\alpha}$ and $\sum F_2$-IsoP concentrations than did the pair-fed rats. Abbreviations: AA, arachidonic acid. Data are presented as mean±S.E.

Table 3
Antioxidant status of the Sprague–Dawley rats fed with a zinc-adequate diet, a zinc-deficient diet and a pair-fed diet for 21 days

	Zinc-adequate diet ($n=12$)	Zinc-deficient diet ($n=12$)	Pair-fed diet ($n=12$)	P
FRAP (μmol/L)	180±19[a]	154±25[b]	184±25[a]	.039
Plasma vitamin C (μmol/L)	43.6±9.2[a]	28.6±6.1[b]	28.5±11.0[b]	.003
Plasma uric acid (μmol/L)	12.8±2.1[a]	6.6±0.7[b]	11.8±2.8[a]	<.0001
Plasma α-tocopherol (μmol/L)	23.5±2.9[a]	15.3±1.6[b]	14.3±2.3[b]	<.0001
Plasma γ-tocopherol (μmol/L)	0.30±0.10[a]	0.21±0.05[b]	0.17±0.03[b]	<.0001
Hepatic α-tocopherol (nmol/g)	34.6±6.1[a]	21.5±2.6[b]	27.0±4.1[c]	<.0001
Hepatic γ-tocopherol (nmol/g)	0.60±0.12[a]	0.44±0.16[b]	0.62±0.11[a]	<.0001

Statistical analysis was performed using one-way ANOVA. The P values shown in the table are the main effects of the analysis. Values (mean±S.D.) across a row with different superscript letters are significantly different ($P<.05$) as determined by Tukey's post hoc analysis. At the completion of the investigation, the zinc-deficient rats had significantly lower FRAP and uric acid concentrations. Plasma vitamin C as well as plasma α-tocopherol and γ-tocopherol were also reduced in the zinc-deficient rats, but similar alterations were observed among the pair-fed rats. Lastly, the zinc-deficient rats had 38% lower hepatic α-tocopherol and 27% lower hepatic γ-tocopherol concentrations as compared with the zinc-deficient rats.

incubation with a primary antibody at 4°C and then an additional incubation for 1 h with a secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA). For α-tocopherol transfer protein (TTP), a rabbit antiserum was generated against the C-terminal 21 amino acids of the rat α-TTP sequence [27], which was a generous gift from Robert Farese (Gladstone Institute, University of California–San Francisco, San Francisco, CA, USA). Antibody dilutions were as follows: α-TTP, 1:2000; anti-rat cytochrome *P*450 (CYP) 3A2, 1:4000 (Chemicon); and anti-human CYP4F2 (a generous gift from Jerome M. Lasker, Institute for Biomedical Research, Lackensack University Medical Center) and anti-rat β-actin, 1:4000 (Sigma, St. Louis, MO, USA). Detection was conducted using a Western Lightning Chemiluminescence Reagent Plus system (PE Life Sciences, Boston, MA, USA) with image analysis on an AlphaInnotech photodocumentation system. Image quantification and densitometry were determined using NIH ImageJ.

2.6. Statistical analysis

Statistical analysis was performed with the use of GraphPad PRISM (version 4.0; GraphPad Software, San Diego, CA, USA). One-way ANOVA was used for all comparisons between dietary treatments, with Tukey's post hoc test when appropriate. Data were considered statistically significant at $P<.05$. All data are reported as mean±S.D. unless otherwise indicated.

3. Results

3.1. Dietary intakes and body weights

Rats fed the zinc-adequate diet consistently gained weight throughout the experiment (Fig. 1A). Dietary zinc restriction resulted in reduced food intake and stunted growth. In addition, rats fed the zinc-deficient diet also showed other signs of zinc deficiency, including loss of hair, decreased activity and increased agitation. At the completion of the experiment, rats fed the zinc-deficient diet had body weights (111±6 g) that were 49% and 24% lower ($P<.0001$) than those of the zinc-adequate (224±12 g) and pair-fed (150±4 g) rats, respectively. Despite matched caloric intakes (Fig. 1B) between the rats fed the zinc-deficient diet and those that received the pair-fed diet, those that were zinc restricted (<0.5 mg/kg) had exacerbated weight loss ($P<.0001$) such that the zinc-deficient rats weighed 26% less than did the pair-fed rats at the completion of the investigation.

3.2. Plasma and hepatic zinc concentrations

Plasma zinc concentrations are generally regarded as a poor biomarker for assessing in vivo zinc status [28]. As such, we observed no significant difference ($P>.05$) in plasma zinc concentrations between the dietary treatments (Table 2). However, hepatic zinc concentrations were significantly lower ($P<.05$; Table 2) in the rats fed with the zinc-deficient diet as compared with the rats fed with the zinc-adequate diet and those that received the pair-fed dietary treatment. Thus, on the basis of hepatic zinc concentrations, the observed stunting in body weight gain and the additional zinc deficiency symptoms (Fig. 1), the rats were confirmed to be zinc deficient.

3.3. Plasma F_2 IsoPs

F_2 IsoPs are generated from free radical-mediated oxidation of arachidonic acid and are considered to be the gold standard measure of oxidative stress [29]. To evaluate the extent to which zinc deficiency increases lipid peroxidation, we measured specific IsoP species along with arachidonic acid by LC/MS/MS according to procedures recently developed in our laboratory [17].

We observed that dietary zinc restriction and pair feeding altered arachidonic acid metabolism such that the animals had significantly lower arachidonic acid concentrations ($P<.0001$; Fig. 2A) than did the zinc-adequate animals. A reduction in arachidonic acid has been reported by other groups and is likely a function of reductions in delta desaturase activity [30–34].

Since arachidonic acid is the precursor fatty acid of the IsoPs, we used plasma arachidonic acid values to normalize plasma F_2-IsoP concentrations. Upon normalization, we observed that the zinc-deficient rats had significantly higher 8-iso-15(*R*)-$PGF_{2\alpha}$ ($P<.0001$), 8-iso$PGF_{2\alpha}$ ($P<.0001$) and

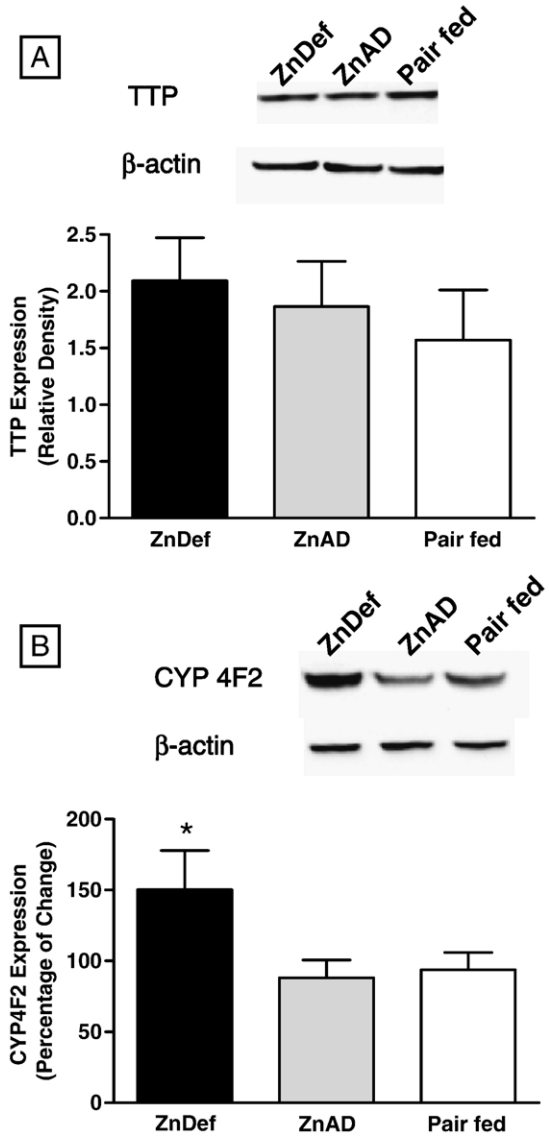

Fig. 3. Zinc deficiency increased hepatic CYP4F2 expression with no change in hepatic α-TTP. Liver lysates were prepared from the zinc-deficient, zinc-adequate and pair-fed ($n=12$ per group) rats, and Western blot analyses for hepatic α-TTP and CYP4F2 were performed. Representative blots and corresponding densitometry (mean±S.E.) findings are illustrated. In Panel A (α-TTP expression), relative density was normalized to β-actin. In Panel B (CYP4F2 expression), the densitometry of CYP4F2 expression normalized to β-actin calculated versus the percentage of change to zinc adequacy. No significant change in α-TTP levels was observed, but zinc deficiency significantly increased ($P<.05$) CYP4F2 protein levels.

$\sum F_2$ IsoP ($P<.0001$) levels as compared with the pair-fed rats (Fig. 2B–E). Even without normalization to arachidonic acid, there was a significant increase ($P<.05$) in all IsoP measures in the zinc-deficient rats as compared with the pair-fed animals. The nonnormalized IsoP values for the zinc-deficient and pair-fed animals were as follows: 15(*R*)-$PGF_{2\alpha}$, 215.5±14.65 versus 182.0±12.75 pg/ml; 8-iso-$PGF_{2\alpha}$, 210.6±11.49 versus 180.1±10.66 pg/ml; and $\sum F_2$ IsoP, 1446±69.41 versus 1245±58.14 pg/ml. At present, it is unclear why the pair-fed rats had slightly higher plasma values ($P<.05$) of 8-iso$PGF_{2\alpha}$, $PGF_{2\alpha}$ and total IsoP as compared with the zinc-adequate rats. However, from the available data, we could speculate that the severe dietary restriction (~40–50%) among the pair-fed rats (Fig. 1A and B), which blunted growth, induced stresses that resulted in slightly elevated lipid peroxidation. Nonetheless, our data suggest that zinc deficiency markedly increased oxidative stress as measured by several unique IsoP species.

3.4. Antioxidant status: FRAP, vitamin C, uric acid and vitamin E

Zinc may have antioxidant effects [35], but the extent to which zinc restriction alters in vivo antioxidant status remains unclear. In an attempt to elucidate the mechanism by which zinc deficiency increases oxidative damage, we evaluated the antioxidant status of the rats.

Plasma FRAP provides a relative assessment of plasma antioxidant status. Zinc-deficient rats had significantly lower ($P<.05$) FRAP concentrations than did rats fed with the zinc-adequate diet and those that received the pair-fed diet, whereas no significant difference was observed between the zinc-adequate rats and the pair-fed ones ($P>.05$; Table 3). Since the major determinants of FRAP are uric acid and ascorbic acid concentrations [18], we assessed these specific antioxidants in the plasma. Zinc deficiency resulted in an approximately 50% reduction ($P<.0001$) in plasma uric acid (Table 3) as compared with the concentrations in the zinc-adequate and pair-fed rats. However, plasma ascorbic acid concentrations were decreased ($P=.003$) in the zinc-deficient and pair-fed rats as compared with the concentration in the zinc-adequate rats (Table 3). Thus, these data suggest that zinc deficiency likely did not alter ascorbic acid concentrations ($P>.05$).

Although FRAP is not significantly dependent on vitamin E concentrations [18], we assessed plasma and hepatic vitamin E (α-tocopherol and γ-tocopherol) to gain greater understanding on whether decreases in vitamin E could explain, at least in part, the observed elevations in F_2 IsoP. The zinc-deficient and pair-fed rats had lower ($P<.0001$) plasma α-tocopherol and γ-tocopherol concentrations than did the rats fed with the zinc-adequate diet (Table 3). However, hepatic α-tocopherol and γ-tocopherol concentrations were significantly reduced by zinc deficiency alone (Table 3). Specifically, the zinc-deficient rats had hepatic α-tocopherol concentrations that were 38% lower as compared with those in the zinc-adequate rats but only 21% lower as compared with those in the pair-fed rats, suggesting that zinc deficiency further enhanced any dietary induced alteration in hepatic α-tocopherol. Similarly, hepatic γ- tocopherol was significantly reduced among the zinc-deficient rats by 27% as compared with the zinc-adequate rats, but no difference was observed between the pair-fed and zinc-adequate rats; these data indicate that zinc deficiency alone decreased hepatic γ-tocopherol. Collectively, these data support the hypothesis that zinc deficiency may compromise hepatic

vitamin E status, which in turn contributes to enhanced oxidative damage.

3.5. Regulation of hepatic vitamin E

Hepatic vitamin E concentrations are regulated by the secretion of tocopherols into plasma or bile or by the metabolism of tocopherols to CEHCs [36]. The hepatic α-TTP facilitates the secretion of α-tocopherol from the liver to the plasma [37,38]. Based on the data demonstrating selective loss of tocopherols in the liver, we explored if zinc deficiency would decrease the hepatic α-TTP and thus limit the secretion of α-tocopherol into the plasma. We observed no significant change between the dietary treatments with respect to α-TTP levels (Fig. 3A). This observation is consistent with the lack of change in the plasma α-tocopherol concentrations that we observed between the zinc-deficient rats and the pair-fed rats (Table 3). CYPs of the CYP3A or 4F type are thought to be involved in the metabolism of tocopherols to CEHCs [39–42]. Therefore, we investigated the extent to which zinc deficiency may have decreased tocopherol concentrations through increased metabolism of tocopherols. We observed no significant alteration in the hepatic protein levels of CYP3A (data not shown). However, as illustrated in Fig. 2B, hepatic CYP4F2 concentrations were significantly increased by approximately 50% among the zinc-deficient rats as compared with the zinc-adequate and pair-fed rats. Despite the significant elevation in hepatic CYP4F2 with zinc deficiency, we did not observe a significant increase in hepatic γ-CEHC concentrations. In fact, the zinc-deficient rats (0.11±0.02 nmol/g) had significantly lower ($P<.05$) hepatic γ-CEHC concentrations as compared with the zinc-adequate rats (0.19±0.08 nmol/g), although there was no significant difference between the zinc-deficient rats and the pair-fed rats. However, further supporting our findings that zinc deficiency did not enhance tocopherol metabolism was the fact that hepatic α-CEHC was consistently below the detection limits of the LC/MS (~20-fmol injection) among all dietary treatments. Lastly, CYP4F2 is also known to be involved in the metabolism of arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) [43], which may provide partial explanation for our observed reductions in the arachidonic acid concentrations.

4. Discussion

In this investigation, zinc deficiency in rats compromised antioxidant status and significantly increased oxidative damage as assessed by specific plasma F_2-IsoP species, suggesting that zinc has an in vivo antioxidant function. Consistent with enhanced oxidative stress induced by zinc deficiency, we observed significant reductions in plasma FRAP and uric acid, lower hepatic vitamin E and elevated hepatic CYP42 protein levels.

An antioxidant function for zinc has been previously suggested; however, the mechanisms are unclear. CuZn superoxide dismutase (CuZnSOD) is a critical enzyme for oxygen free radical defense and is a zinc-containing enzyme. Thus, several researchers focused on CuZnSOD activity as a central mechanism leading to oxidative stress with zinc deficiency. However, researchers have found that there is little or no change in CuZnSOD activity with zinc deficiency as compared with pair feeding [12]. In some cases, there has been an increase in tissue CuZnSOD activity with zinc deficiency [9,44]. Thus, the oxidative stress that is apparent in zinc deficiency cannot be solely attributed to loss of CuZnSOD. In an effort to understand the underlying mechanisms of zinc deficiency-induced oxidative damage, we measured several antioxidant markers to determine the extent to which zinc deficiency compromises antioxidant status. We determined that zinc deficiency significantly decreased FRAP concentrations, which suggested an overall decrease in nonenzymatic antioxidant defenses. Given the lack of specificity of FRAP, we measured specific antioxidant concentrations and observed that plasma uric acid concentrations, but not plasma ascorbic acid concentrations, were significantly decreased among rats with zinc deficiency independent of reduced dietary food intake (Table 3). Although excessively high uric acid concentrations are considered to be deleterious to humans [45], physiologic levels are recognized to have an important antioxidant function [46–48]. Consistent with this notion, an inverse relationship between plasma F_2-IsoPs and uric acid concentrations was observed in congestive heart failure patients [49] — a human population often described as having a greater magnitude of oxidative stress. Thus, the reductions in plasma uric acid may provide partial explanation for the increased F_2 IsoPs among zinc-deficient rats. Although the regulatory mechanism by which zinc regulates uric acid concentrations was beyond the scope of our investigation, it is plausible that zinc deficiency may have increased uric acid utilization via an antioxidant mechanism or that zinc deficiency may have increased uricase activity, as has been described in vitro [50]. Certainly, this is an area that warrants further investigation.

To further understand the relationship between zinc deficiency and antioxidant status, we evaluated plasma and hepatic α-tocopherol and γ-tocopherol. Although our data indicate that there was no specific zinc-mediated decrease in plasma tocopherols, we observed a significant reduction in hepatic α-tocopherol and γ-tocopherol among the zinc-deficient rats as compared with the zinc-adequate and pair-fed rats (Table 3). These observations suggest that the liver, as compared with plasma, had compromised vitamin E status coinciding with zinc deficiency. Importantly, others have demonstrated that vitamin E supplementation effectively reduced oxidative stress as measured by F_2 IsoPs [51,52]. Thus, it is likely that the increased plasma F_2 IsoPs in the present study may be partially attributed to the reduced hepatic vitamin E concentrations.

It has been suggested that plasma vitamin E concentrations may not be adequate to assess vitamin E status even

in models of oxidative stress [53,54]. This is exemplified by data, including our own, that cigarette smoke-induced oxidative stress in humans does not consistently decrease plasma vitamin E concentrations [20,55]. Thus, alternative biomarkers of vitamin E status are necessary since plasma vitamin E measurements may lack the necessary specificity. Therefore, hepatic α-CEHC and γ-CEHC were evaluated to gain an understanding on whether the reductions in hepatic tocopherols were attributed to increased *P*450-mediated vitamin E metabolism. Contrary to our hypothesis, our data indicate that zinc deficiency did not increase the metabolism of tocopherols to CEHCs since hepatic γ-CEHC concentrations were not elevated and neither was α-CEHC detectable. However, it should be noted that the protein levels of CYP4F2 were significantly increased in the zinc-deficient rats and that the CYP3A2 levels were unchanged — two *P*450 isoforms that are involved in the metabolism of vitamin E [39–42]. Thus, the specific mechanism by which zinc may be involved in vitamin E metabolism warrants further investigation.

CYP4F2 is also involved in the omega-hydroxylation of arachidonic acid to 20-HETE [43]. This additional role aids in the explanation for the reduced plasma arachidonic acid concentrations that we observed among the zinc-deficient rats. Alternatively, a reduction in delta desaturase activity may also explain the reductions in plasma arachidonic acid concentrations [30–34]. Interestingly, the pair-fed rats also had CYP4F2 levels and hepatic zinc concentrations similar to those of the zinc-adequate rats but had plasma arachidonic acid levels that were reduced. Thus, it is likely that these rats had lower arachidonic acid concentrations due to the severe dietary restriction imposed on them. Regardless of the mechanisms by which zinc alters arachidonic acid concentrations, it proved important to simultaneously measure arachidonic assay and the F_2-IsoP species to accurately assess the oxidative stress in the present study.

Given that *P*450-mediated vitamin E metabolism was not enhanced by zinc deficiency, the most likely mechanism for decreased hepatic tocopherol concentrations is via enhanced tocopherol oxidation consistent with vitamin E's antioxidant function [55]. The lack of increase in CEHC by zinc deficiency is not surprising given the fact that cigarette smoking, an established model of oxidative stress in humans, did not result in increased CEHC production [54,55]. An alternative explanation for reduced hepatic vitamin E concentrations could be attributed to the fact that zinc deficiency decreased vitamin E absorption [56,57].

Given the reductions in hepatic tocopherols, we sought to determine the effects of zinc deficiency on mechanisms regulating tocopherol distribution. The α-TTP facilitates the secretion of vitamin E from the liver to the plasma via VLDL through a mechanism that is incompletely understood [58]. Given the involvement of zinc in numerous enzymes, proteins, transcription factors and metalloproteins, we hypothesized that the α-TTP would be regulated by zinc status. Contrary to our hypothesis, we observed no significant change in the protein levels of α-TTP (Fig. 2A). Thus, it appears that zinc status does not regulate vitamin E status through the actions of the α-tocopherol protein.

Collectively, the findings of our study provide additional insights into the function of zinc in maintaining antioxidant status in the body. Although zinc itself may have no direct reactive oxygen species-scavenging abilities, the specific mechanism by which it functions as an antioxidant appears to be indirect and multifactorial. Zinc deficiency induces complex physiologic changes, including enhanced CYP4F2 protein levels, which appear to be important in the metabolism of arachidonic acid, and/or an increase in reactive oxygen species, which may contribute to the enhanced formation of F_2 IsoP. Additionally, zinc deficiency may decrease vitamin E bioavailability [56] and/or increase vitamin E oxidation consistent with its peroxyl radical-scavenging ability, which may also exacerbate an animal's susceptibility to oxidative stress. Others have also shown loss of glutathione status with zinc deficiency, which could contribute to increased susceptibility [59–61]. Clearly, many of the underlying mechanisms by which zinc deficiency alters antioxidant status warrant further investigation since a significant proportion of the American population has compromised zinc status [3].

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