ELSEVIER

In vitro zinc stimulation of angiotensin-converting enzyme activities in human plasma

Tsunenobu Tamura, Robert L. Goldenberg,* Kelley E. Johnston, Larry E. Freeberg, Mary B. DuBard,* and Elizabeth A. Thomas†

*The Departments of Nutrition Sciences and *Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, AL USA; †Department of Human Nutrition and Foods, Virginia Polytechnic Institute and State University, Blacksburg, VA USA*

We evaluated plasma angiotensin-converting enzyme (ACE) activity and in vitro zinc stimulation of ACE as a method to assess zinc nutriture in two groups of humans. These included a total of 135 indigent African-American women who participated in a double-blind trial to evaluate the effect of oral zinc supplementation (25 mg/day as zinc sulfate) on pregnancy outcome and 15 healthy young males who volunteered in a study to evaluate the effect of low-zinc intake (0.5 mg of zinc per day for 12 days) on the concentration of erythrocyte metallothionein under controlled metabolic conditions. In the first group, increased birth weight and head circumference of newborns by maternal zinc supplementation led to the conclusion that inadequate maternal zinc nutriture existed. However, there were no differences in the activities of plasma ACE and ACE stimulation by the in vitro addition of zinc between the zinc-supplemented and placebo groups. Both of these values remained unchanged during pregnancy. In the second group, plasma ACE activity remained unchanged after a low-zinc diet for 12 days although erythrocyte metallothionein and zinc concentrations declined. We conclude that these tests are insensitive indicators of zinc nutriture in humans. (J. Nutr. Biochem. 7:55–59, 1996.)

Keywords: zinc; angiotensin-converting enzyme; plasma; in vitro stimulation; humans; pregnancy; African Americans

Introduction

Although more than three decades have passed since the first discovery of human zinc deficiency,[1] a sensitive and specific method to evaluate zinc nutriture in humans accurately remains to be developed.[2] Reeves and O'Dell[3] reported that the activities of serum angiotensin-converting enzyme (ACE, EC 3.4.15.1) are significantly lower in zinc-deficient rats than in zinc-supplemented controls, and these activities in serum obtained from zinc-deficient rats were fully restored by the in vitro addition of zinc. A similar observation was reported by German researchers indicating that the activities of ACE in plasma obtained from zinc-deficient rats were significantly increased by the in vitro addition of zinc while such an increase was not observed in zinc-sufficient controls.[4,5] Using pregnant guinea pigs, Apgar and Everett[6] reported that plasma ACE activities were lower in zinc-deficient animals than in zinc-supplemented controls. The activities were more greatly stimulated by the in vitro addition of zinc in zinc-deficient guinea pigs than controls. They proposed that the change in ACE activities with the in vitro addition of zinc may be useful as an indicator of zinc nutriture. We reported similar findings using plasma samples obtained from zinc-deficient rats; however, such findings were not observed in other tissues such as the lung, testis, and aorta.[7]

Recently, a group of Japanese investigators suggested that plasma ACE stimulation by the in vitro addition of zinc is a sensitive indicator of zinc nutriture among patients receiving home-total-parenteral nutrition providing insufficient zinc.[8] However, there are conflicting reports as to whether the in vitro addition of zinc increases ACE activities in plasma/serum samples from humans without com-

Address reprint requests to Dr. T. Tamura at the Department of Nutrition Sciences, University of Alabama at Birmingham, 218 Webb Building, UAB Station, Birmingham, AL 35294-3360, USA.
Received May 4, 1995; accepted August 25, 1995.

655 Avenue of the Americas, New York, NY 10010

0955-2863/96/$15.00
SSDI 0955-2863(95)00167-0

promised zinc nutriture.[9–13] Although Roulston and Allan[11] reported that there was an activation of ACE by the in vitro addition of zinc, others failed to demonstrate such an increase in ACE activities by the in vitro addition of zinc to human plasma/serum.[9,10,12,13] Some investigators have even reported an inhibition of ACE activities.[9,10] Furthermore, Bakan et al.[14] reported a significant positive correlation between serum ACE activities and zinc concentrations in patients with lung cancer as well as in healthy controls while Roulston and Allan[11] found no such correlation. Ruz et al.[15] reported that plasma ACE activities were not affected in 15 male volunteers with mild-zinc depletion induced by feeding a diet containing 4.0 mg of daily zinc for 6 weeks following 1 week of a low-zinc diet containing 0.6 mg of zinc. Johnson et al.[16] reported that in 11 male volunteers, plasma ACE activities were significantly higher during two periods of 35 days when they were fed diets containing either 1.0 or 2.0 mg of daily zinc than during a period of 35 days when they received a daily zinc intake of 10 mg.

ACE is a chloride- and zinc-dependent enzyme that cleaves histidylleucine from angiotensin I to form a potent vasoconstrictor, angiotensin II; therefore, it plays an important role in the renin-angiotensin system.[17–19] Ethylendaimine-tetraacetic acid (EDTA) inhibits the activities of ACE in various tissues which can be reversed by the addition of zinc. The binding of zinc to ACE is known to be weak as compared with other zinc-enzyme complexes.[18] We hypothesized that such a property of ACE may be suitable for a functional test to assess zinc nutriture in humans; therefore, we undertook this study to evaluate whether stimulation of plasma ACE activities by the in vitro addition of zinc correlates with zinc nutriture in humans. We used two populations who presumably had inadequate zinc nutriture. These included a group of pregnant women who participated in a double-blind trial to evaluate the effect of zinc supplementation on pregnancy outcome[20] and a group of healthy males who volunteered in a study to evaluate the response of erythrocyte metallothionein to low-zinc intake.[21]

Methods and materials

Subjects

Study 1. A total of 135 subjects in the present study were selected from 580 indigent African-American pregnant women who participated in a randomized double-blind trial to evaluate the effect of oral zinc supplementation (25 mg of zinc/day as zinc sulphate) on pregnancy outcome.[20] The subjects who received their prenatal care at the Jefferson County Health Department were selected based on their plasma zinc levels being below an estimated median adjusted for gestational age. The median values were established based on the data from 176 women with a similar socioeconomic background who were enrolled at the clinics and ranged from 11.3 μmol/L at 6 weeks to 9.5 μmol/L at 22 weeks of gestation. Their mean age was 23.3 years old with a range between 13 and 39. The subjects were randomized to receive either zinc supplementation (n = 70) or placebo (n = 65) at a mean of 18.7 weeks of gestation. The subjects in both groups were offered a daily multivitamin/mineral tablet not containing zinc (Mission Pharmacal, San Antonio, TX USA) throughout pregnancy. The compliance of taking the supplements was about 78% in both groups as assessed by pill counting. The concentrations of plasma and erythrocyte zinc, the activities of plasma ACE and ACE stimulation by the in vitro addition of zinc were monitored throughout pregnancy. Women with medical risks for having reduced or excessive birth weight (i.e., hypertension, renal disease, diabetes, etc.) were excluded from this trial. The study was approved by the Institutional Review Board of the University of Alabama at Birmingham and informed consent was obtained from each subject.

Non-fasting blood samples were obtained in trace mineral–free evacuated tubes containing heparin (Vacutainer #6527, Beckton-Dickinson, Rutherford, NJ) prior to the randomization at a mean of 18.7 weeks of gestation and at mean gestations of 25.9 and 36.5 weeks and at delivery (mean 39.2 weeks). Blood samples were kept under refrigeration until plasma separation and aliquots of whole blood and plasma samples were stored at −70°C until analyses. Every effort was made to minimize the contamination of zinc.[22]

Study 2. A total of 15 healthy male volunteers between 22 and 35 years old participated in the study to evaluate the effect of dietary zinc on erythrocyte metallothionein.[21] The subjects were given diets containing zinc (either 3.2, 7.2, or 15.2 mg/day) using a 4-day-cycle menu for 50 days at the Clinical Research Center at the University of Florida. Subsequently they received a diet providing only 0.55 mg of zinc daily for 12 days. The concentrations of plasma and erythrocyte zinc were monitored throughout the study. The study was approved by the Institutional Review Board of the University of Florida, and all subjects signed informed consent.

Fasting heparinized blood samples were obtained using trace mineral–free tubes at days 51 and 62. After separation of plasma, samples were kept at −20°C until they were shipped on dry ice from Gainesville to Birmingham for ACE assay.

Methods

Plasma ACE activities were measured by the method originally described by Ryan[23] using an artificial substrate; [^{3}H]-hippuryl-glycyl-glycine ([^{3}H]-HGG, specific activity, 20.5 GBq/mmol, Amersham, Arlington Heights, IL USA). Briefly, the reaction mixture contained 300 mmol/L of NaCl; [^{3}H]-HGG, 22 pmol/L (approximately 10,000 dpm); 20 mmol/L of potassium phosphate buffer, pH 8.0; and 20 μL of plasma as the enzyme source in a final volume of 50 μL with or without 17 μmol/L of $ZnSO_4$. The incubation was initiated by the addition of buffer/substrate after a 5 min preincubation of the enzyme with zinc or H_2O and carried out at 37°C for 1 hr. The reaction was terminated by adding 50 μL of 1 mol/L of HCl, and hydrolyzed [^{3}H]-hippuric acid was extracted by vortexing into 500 μL of ethyl acetate. After centrifugation, the ethyl acetate layer (400 μL) was counted (1219 Rackbeta counter, LKB Instruments, Gaithersburg, MD USA) and ACE activities were expressed as nanomoles of [^{3}H]-hippuric acid produced per hour per liter of plasma. The ACE determinations were carried out in duplicate. ACE stimulation by the in vitro addition of zinc was defined as a ratio of ACE activity with the in vitro addition of zinc to ACE without the addition of zinc.[7]

To monitor daily variations in the ACE assay, repeated analyses of ACE activities with and without the in vitro addition of zinc were performed using dialyzed human plasma or pooled plasma obtained from zinc-deficient rats. Dialyzed human plasma was prepared as previously described.[7] The activity of ACE in these preparations determined with the in vitro addition of zinc was stable for at least 12 months at −70°C, and the coefficient of the interassay variation of ACE activity was approximately 6%.

Plasma and erythrocyte zinc concentrations were determined by flame atomic absorption spectrophotometry; plasma samples

with visible hemolysis were excluded from this analysis.[22,24] Statistical analyses were performed using the Statistical Analysis System.

Results

Study 1

In pregnant women, neither ACE stimulation by the in vitro addition of zinc nor ACE activities were significantly different between the zinc-supplemented and placebo groups throughout pregnancy and at delivery. ACE stimulation by the in vitro addition of zinc and plasma ACE activities remained essentially the same from 19 weeks of gestation to delivery (*Figures 1A* and *1B*). There was a significant negative correlation between plasma ACE activities and ACE stimulation by the in vitro addition of zinc in each group. This finding indicates that the higher the plasma ACE activities, the lower the ACE stimulation by the in vitro addition of zinc. Plasma zinc concentrations remained higher in the zinc-supplemented group than in the placebo group after the initiation of zinc supplementation (*Figure 2A*). The difference in plasma zinc concentrations between the two groups was significant at 26 weeks of gestation ($P = 0.02$); however, this was not significant at 36 weeks of gestation. The concentrations of erythrocyte zinc remained essentially the same in both groups and there was no difference between the two groups throughout pregnancy (*Figure 2B*).

Study 2

In the study using healthy male volunteers, there were no significant changes in plasma ACE activities nor in ACE stimulation by the in vitro addition of zinc during the period when the volunteers were given a low-zinc diet for 12 days (*Table 1*). As previously reported, in these subjects, erythrocyte zinc and metallothionein concentrations decreased significantly while plasma zinc did not change.[21]

Discussion

In Study 1, the supplementation of zinc influenced neither ACE stimulation by the in vitro addition of zinc nor plasma ACE activities in a group of 135 pregnant women randomly selected from a total of 580 subjects who participated in a double-blind trial of zinc supplementation (*Figure 1*). The population of total subjects who were selected based on

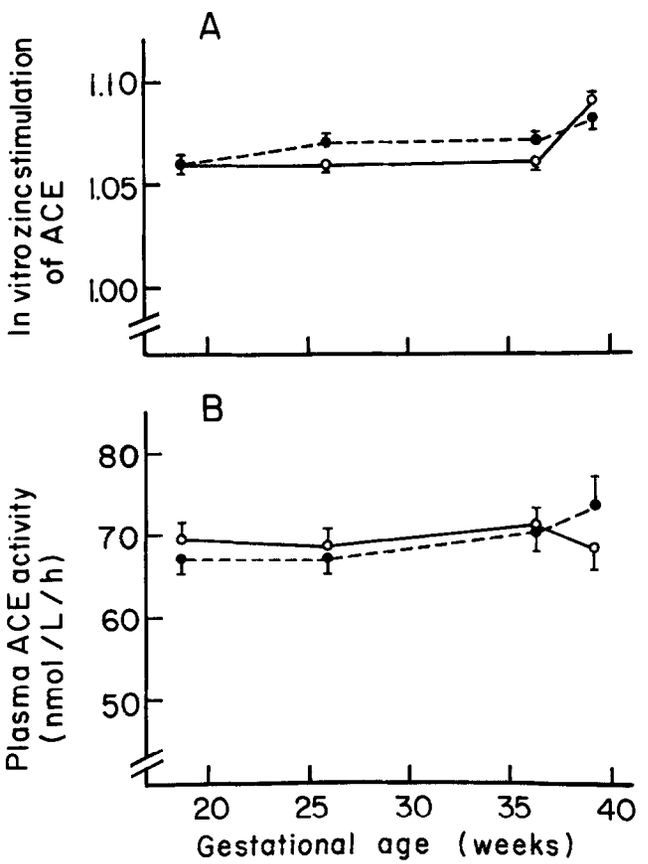

Figure 1 Changes in plasma angiotensin-converting enzyme (ACE) stimulation by the *in vitro* addition of zinc (A) and plasma ACE activities (B) during pregnancy. Vertical lines represent SEM. The solid line represents the zinc-supplemented group and the broken line the placebo group.

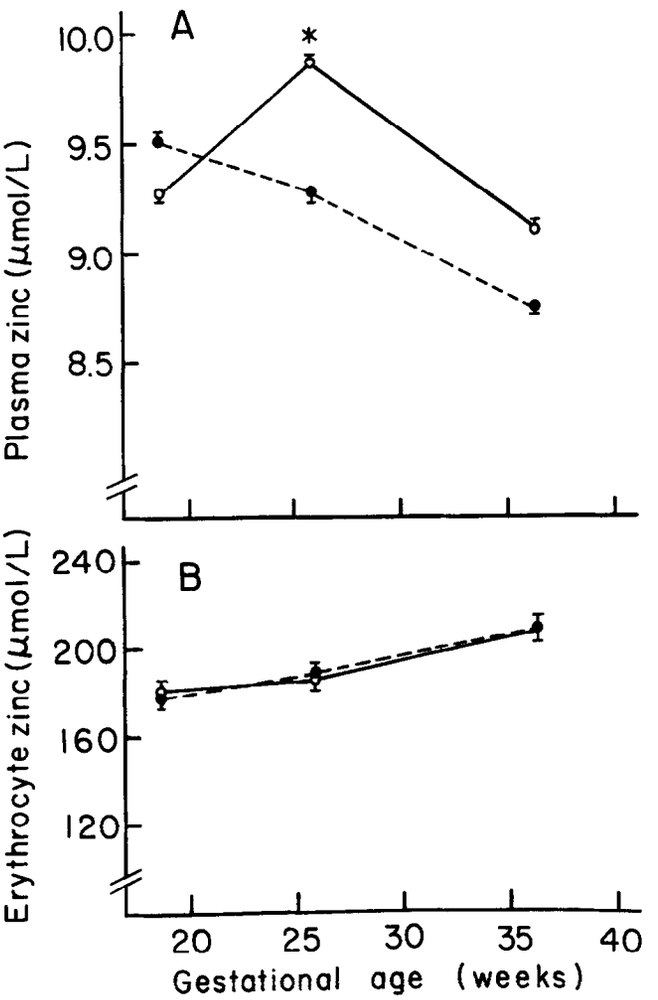

Figure 2 Changes in plasma (A) and erythrocyte (B) zinc concentrations during pregnancy. The vertical lines represent SEM. The solid line represents the zinc-supplemented group and the broken line the placebo group. *The difference between the zinc-supplemented and placebo groups was significant ($P = 0.02$).

Table 1 The mean plasma angiotensin-converting enzyme (ACE) activity, stimulation by the in vitro addition of zinc of ACE, and the plasma and erythrocyte zinc concentration in healthy male volunteers before and after receiving a low-zinc diet for 12 days

Analysis	Before	After
ACE activity (nmol/L/hr)	143.7 ± 31.9	124.5 ± 34.4
ACE stimulation by the in vitro addition of zinc	1.10 ± 0.08	1.05 ± 0.07
Plasma zinc concentration (μmol/L)	15.0 ± 1.4	15.9 ± 2.4
Erythrocyte zinc concentration (μmol/L of lysate)	117.6 ± 5.8*	74.4 ± 3.1*

Values are means ± SD. Plasma and erythrocyte zinc concentrations were from Ref. 22. The erythrocyte lysate was prepared by diluting washed cells using double-distilled water (1:1.4).
*Values are significantly different between before and after feeding of a low-zinc diet.[22]

their plasma zinc concentrations being in the lower 50th percentile at about 19 weeks of gestation responded to zinc supplementation in terms of pregnancy outcome including birth weight and head circumference of infants[20]; therefore, we assumed that subjects selected for the present study had prior inadequate zinc nutriture. In Study 2, we determined ACE stimulation by the in vitro addition of zinc and plasma ACE activities among male volunteers who participated in a study evaluating the response of erythrocyte metallothionein concentrations to low-zinc intake under well-controlled conditions.[21] There was no effect of a low-zinc diet (0.55 mg per day) on ACE stimulation by the in vitro addition of zinc and plasma ACE activities among these volunteers (*Table 1*), although these subjects had significant decreases in erythrocyte zinc and metallothionein concentrations in response to 12 days of a low-zinc diet.[21] Our findings are consistent with the observation of Ruz et al.[15] who reported that there was no effect of mild-zinc depletion on plasma ACE activities among young male adults. However, our data are in conflict with the recent findings by Nezu et al.[8] who reported a significant increase in ACE stimulation by the in vitro addition of zinc in zinc-depleted patients receiving home-total-parenteral nutrition. The reason for the difference between our data and those of Nezu et al.[8] is not known at present.

It has been well established that ACE stimulation by the in vitro addition of zinc was significantly higher in plasma obtained from zinc-deficient rats than controls.[3–5,7] Our findings suggest that the characteristics of ACE stimulation by the in vitro addition of zinc of plasma obtained from human subjects with inadequate zinc nutriture were different from those in plasma obtained from severely zinc-deficient rats. Although the reason for this difference is unknown, it may be that: the degree of zinc deficiency in the subjects in the present study was not as severe as that of zinc-deficient rats fed a low-zinc diet for an extended period; the production (or induction) of ACE apoenzyme is less responsive to inadequate zinc nutriture in humans than in rats. It is apparent that the production of plasma ACE apoenzyme is significantly increased in zinc-deficient rats, since ACE activities were higher than those of control rats with the in vitro addition of zinc[5,7]; the binding of human plasma ACE to zinc added in vitro is different from that of rat plasma ACE. However, this possibility is unlikely, since ACE activities in dialyzed-human plasma were restored by the in vitro addition of zinc[7]; and the origin(s) of plasma ACE is unknown in these two species. If these are in different tissues, the characteristics of plasma ACE may be different.

There are conflicting reports as to whether plasma/serum ACE activities change during pregnancy.[25–28] Oats et al.[26] reported that plasma ACE activities increased after 30 weeks of gestation until term. However, others reported that serum ACE activities did not change throughout pregnancy,[25,27,28] and our findings are in agreement with those. It is generally accepted that the activities of plasma/serum ACE are low throughout pregnancy as compared with non-pregnant controls; however, the mechanisms responsible for low activities are unknown.

There are three genotypes of an insertion/deletion polymorphism that affect plasma ACE activities.[29–32] Therefore, we anticipated that the determination of the ratio of plasma ACE activity with the in vitro addition of zinc to without the addition of zinc might be suitable for the assessment of zinc nutriture. This ratio would eliminate the variation of plasma ACE activities due to polymorphism. However, based on our data in two groups of human subjects, the ratio of plasma ACE activities with the in vitro addition of zinc to without the addition of zinc is not an adequate indicator of zinc nutriture among humans. There still remains an urgent need to identify a specific and sensitive method to assess accurately zinc nutriture in humans.

Acknowledgments

This study was supported in part by grants from the National Institutes of Health (HD28119, HD27289, and RR00082), a USDA grant (90-37200-5636), and the Agency for Health Care Policy Research Contract (DHHS 282-92-0055).

References

1 Prasad, A.S. (1991). Discovery of human zinc deficiency and studies in an experimental human model. *Am. J. Clin. Nutr.* **53,** 403–412
2 King, J.C. (1990). Assessment of zinc status. *J. Nutr.* **120,** 1474–1479
3 Reeves, P.G. and O'Dell, B.L. (1985). An experimental study of the effect of zinc on the activity of angiotensin converting enzyme in serum. *Clin. Chem.* **31,** 581–584
4 White, C.L., Pschorr, J., Jacob, I.C.M., von Lutterotti, N., and Dahlheim, H. (1986). The effect of zinc *in vivo* and *in vitro* on the activities of angiotensin converting enzyme and kininase-I in the plasma of rats. *Biochem. Pharmacol.* **35,** 2489–2493
5 Dahlheim, H., White, C.L., Rothemund, J., von Lutterotti, N., Jacob, I.C.M., and Rosenthal, J. (1989). Effect of zinc depletion on angiotensin I-converting enzyme in arterial walls and plasma of the rat. *Miner. Electrolyte Metab.* **15,** 125–129
6 Apgar, J. and Everett, G.A. (1991). Low zinc intake affects maintenance of pregnancy in guinea pigs. *J. Nutr.* **121,** 192–200
7 Tamura, T., Freeberg, L.E., Johnston, K.E., and Keen, C.L. (1994). *In vitro* zinc stimulation of angiotensin-converting enzyme activities in various tissues of zinc-deficient rats. *Nutr. Res.* **14,** 919–928
8 Nezu, R., Kobayashi, H., Fujii, M., Sando, K., Kamiike, W., Takagi, Y., Miyata, M., Matsuda, H., and Okada, A. (1994). Assessment of zinc nutrition using plasma angiotensin converting enzyme activity (ACE) in man. *J. Paraent. Enter. Nutr.* **18,** 21S

9 Huggins, C.G., Corcoran, R.J., Gordon, J.S., Henry, H.W., and John, J.P. (1970). Kinetics of the plasma and lung angiotensin I converting enzymes. *Circl. Res.* **27** (Suppl 1), I93–I101

10 Maguire, G.A. and Price, C.P. (1985). A continuous monitoring spectrophotometric method for the measurement of angiotensin-converting enzyme in human serum. *Ann. Clin. Biochem.* **22,** 204–210

11 Roulston, J.E. and Allan, D. (1987). Studies with an automated kinetic assay for plasma angiotensin-converting enzyme activity and its potentiation by zinc ion. *Clin. Chim. Acta* **168,** 187–198

12 Wong, Y.-W. and Kinniburgh, D.W. (1987). Evaluation of a kinetic assay for angiotensin converting enzyme. *Clin. Biochem.* **20,** 323–327

13 Buttery, J.E. and Stuart, S. (1993). Studies on zinc and angiotensin-converting enzyme. *Clin. Chim. Acta* **217,** 213–216

14 Bakan, N., Bakan, E., Süerdem, M., and Yigitoglu, M.R. (1988). Serum zinc and angiotensin-converting enzyme levels in patients with lung cancer. *BioFactors* **1,** 177–178

15 Ruz, M., Cavan, K.R., Bettger, W.J., Thompson, L., Berry, M., and Gibson, R.S. (1991). Development of a dietary model for the study of mild zinc deficiency in humans and evaluation of some biochemical and functional indices of zinc status. *Am. J. Clin. Nutr.* **53,** 1295–1303

16 Johnson, P.E., Milne, D.B., and Mullen, L. (1990). Experimental zinc deficiency in adult men. *FASEB J.* **4,** A648

17 Ondetti, M.A. and Cushman, D.W. (1982). Enzymes of the renin-angiotensin system and their inhibitors. *Ann. Rev. Biochem.* **51,** 283–308

18 Bünning, P. and Riordan, J.F. (1985). The functional role of zinc in angiotensin converting enzyme: Implications for the enzyme mechanism. *J. Inorg. Biochem.* **24,** 183–198

19 Kleemann, S.G., Keung, W.M., and Riordan, J.F. (1986). Metal binding to angiotensin converting enzyme: Implications for the metal binding site. *J. Inorg. Biochem.* **26,** 93–106

20 Goldenberg, R.L., Tamura, T., Neggers, Y., Copper, R.L., Johnston, K.E., DuBard, M.B., and Hauth, J.C. (1995). The effect of zinc supplementation on pregnancy outcome. *JAMA,* **274,** 463–468

21 Thomas, E.A., Bailey, L.B., Kauwell, G.A., Lee, D.-Y., and Cousins, R.J. (1992). Erythrocyte metallothionein response to dietary zinc in humans. *J. Nutr.* **122,** 2408–2414

22 Tamura, T., Johnston, K.E., Freeberg, L.E., Perkins, L.L., and Goldenberg, R.L. (1994). Refrigeration of blood samples prior to separation is essential for the accurate determination of plasma or serum zinc concentrations. *Biol. Trace Elem. Res.* **41,** 165–173

23 Ryan, J.W. (1984). Angiotensin I converting enzyme (Kininase II). Peptidyl-dipeptide hydrolase, EC 3.4.15.1. In *Methods of Enzymatic Analysis,* 3rd ed., Vol. 5 (H.U. Bergmeyer, ed.), p. 20–34, Verlag Chemie Weinheim, Academic Press, New York, NY USA

24 Smith, J.C. Jr., Butrimovitz, G.P., and Purdy, W.C. (1979). Direct measurement of zinc in plasma by atomic absorption spectroscopy. *Clin. Chem.* **25,** 1487–1491

25 Parente, J.V., Franco, J.G. Jr., Greene, L.J., Meirelles, R.S., and Martinez, A.R. (1979). Angiotensin-converting enzyme: Serum levels during normal pregnancy. *Am. J. Obstet. Gynecol.* **135,** 586–589

26 Oats, J.N., Pipkin, F.B., and Symonds, E.M. (1981). A prospective study of plasma angiotensin-converting enzyme in normotensive primigravidae and their infants. *Br. J. Obstet. Gynaecol.* **88,** 1204–1210

27 Rasmussen, A.B., Pedersen, E.B., Romer, F.K., Johannesen, P., Kristensen, S., Lauritsen, J.G., and Wohlert, M. (1983). The influence of normotensive pregnancy and pre-eclampsia on angiotensin-converting enzyme. *Acta Obstet. Gynecol. Scand.* **62,** 341–344

28 Goldkrand, J.W. and Fuentes, A.M. (1986). The relation of angiotensin-converting enzyme to the pregnancy-induced hypertension-preeclampsia syndrome. *Am. J. Obstet. Gynecol.* **154,** 792–800

29 Rigat, B., Hubert, C., Alhenc-Gelas, F., Cambien, F., Corvol, P., and Soubrier, F. (1990). An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest.* **86,** 1343–1346

30 Tiret, L., Rigat, B., Visvikis, S., Breda, C., Corvol, P., Cambien, F., and Soubrier, F. (1992). Evidence, from combined segregation and linkage analysis, that a variant of the angiotensin I-converting enzyme (ACE) gene controls plasma ACE levels. *Am. J. Hum. Genet.* **51,** 197–205

31 Higashimori, K., Zhao, Y., Higaki, J., Kamitani, A., Katsuya, T., Nakura, J., Miki, T., Mikami, H., and Ogihara, T. (1993). Association analysis of a polymorphism of the angiotensin converting enzyme gene with essential hypertension in the Japanese population. *Biochem. Biophys. Res. Comm.* **191,** 399–404

32 Cambien, F., Costerousse, O., Tiret, L., Poirier, O., Lecerf, L., Gonzales, M.F., Evans, A., Arveiler, D., Cambou, J.P., Luc, G., Rakotovao, R., Ducimetiere, P., Soubrier, F., and Alhenc-Gelas, F. (1994). Plasma level and gene polymorphism of angiotensin-converting enzyme in relation to myocardial infarction. *Circulation* **90,** 669–676