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Ceruloplasmin and Metallothionein Induction by Zinc and 13-*cis*-Retinoic Acid in Rats with Adjuvant Inflammation[1] (42080)

ROBERT J. COUSINS[2] AND MAVIS R. SWERDEL

Department of Food Science and Human Nutrition, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611

Abstract. The induction of ceruloplasmin and metallothionein was investigated in rats with the early inflammatory phase of adjuvant arthritis. When examined at the peak of the acute inflammatory response, 5 days after adjuvant treatment, zinc given daily (2 mg/kg, intraperitoneally) increased serum ceruloplasmin levels by 2.0 times that found in nonarthritic rats and 1.2 times that found in non-zinc-treated arthritic rats. 13-*cis*-Retinoic acid (160 mg/kg, orally) given daily increased serum ceruloplasmin 2.2 and 2.7 times that found in nontreated arthritic rats when given alone and with zinc (2 mg/kg, intraperitoneally), respectively. Reduction in the inflammatory response was measured by weight of the adjuvant-injected paw, 5 days after adjuvant was administered. The reduction in inflammation was 13 and 19–20% for 13-*cis*-retinoic acid and zinc, respectively, when given alone, and between 26 and 31% when the treatments were combined. Zinc markedly increased liver metallothionein levels whereas 13-*cis*-retinoic acid was a much less potent inducer of the protein in liver. The results are discussed in light of the probable physiological roles of both ceruloplasmin and metallothionein.

A universal finding associated with inflammation is hypercupremia. That increase is associated with elevated plasma ceruloplasmin (1–3). The mechanism which accounts for the increase in this acute phase protein may involve a variety of hormones including interleukin-1, epinephrine, and glucocorticoids [(4) a review]. The reason(s) for the increase has not been defined, however. Ceruloplasmin has important roles in redox reactions (5) and as an extracellular scavenger of superoxide radicals (6). This latter property is most easily integrated into a host defense function for the protein (4). Denko has proposed that ceruloplasmin has a protective role during inflammation (2). He demonstrated the anti-inflammatory action of ceruloplasmin in carrageenin-induced limb inflammation. This hypothesis is supported by the demonstration of Moak and Greenwald that hyperoxia elevates serum ceruloplasmin levels (7). They proposed the anti-inflammatory effect of elevated oxygen tensions could be related to ceruloplasmin induction.

Recently, we have been using adjuvant-induced inflammation in rats as a model to evaluate changes in zinc metabolism during experimental inflammation. As a control, we employed 13-*cis*-retinoic acid since Brinckerhoff *et al.* have shown this metabolite of vitamin A decreased the inflammation associated with adjuvant inflammation and arthritis (8). In this paper, we report the results of experiments which show that zinc and 13-*cis*-retinoic acid have singular and additive effects in ameliorating inflammation in this animal model for arthritis and that the effect is concurrent with an increased plasma ceruloplasmin concentration. These data suggest ceruloplasmin may have a beneficial effect on inflammation reduction. In addition, the results support the clinical data of Simkin (9) which imply that zinc has an anti-inflammatory effect in patients with rheumatoid arthritis.

Materials and Methods. *Chemicals.* All chemicals and reagents were from Fisher Scientific Company (Pittsburgh, Pa.) or Sigma Chemical Company (St. Louis, Mo.). 13-*cis*-Retinoic acid was obtained gratis from Dr. Peter Sorter of Hoffman-LaRoche Inc. (Nut-

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[2] To whom all inquiries and reprint requests should be addressed.

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ley, N.J.). Carrier-free ^{109}Cd was from New England Nuclear (Boston, Mass.). All water was glass distilled and deionized. Complete Freund's adjuvant was from Difco Laboratories (Detroit, Mich.).

Animals. Male rats (CD Strain, Charles River, Wilmington, Mass.) weighing about 200 g were maintained as described previously (10). Adjuvant arthritis was induced by injection of 100 μl of complete adjuvant into the left hind paw. Zinc was administered (as $ZnSO_4$ in saline; ip) at 2 mg/kg. 13-*cis*-Retinoic acid was dissolved in peanut oil and given orally by stomach tube at 160 mg/kg (8). Nontreated rats received the appropriate amount of the corresponding vehicle in all experiments.

Measurements. The extent of limb inflammation was measured as the difference in weight between the left (adjuvant treated) and right (nontreated) legs. Each leg was severed at the lateral malleolus immediately after the rats were killed and the weight was recorded to the nearest 0.001 g. Prior experiments showed this method produced data comparable to that obtained by measuring volume changes with a plethysmograph. Ceruloplasmin was measured by the *p*-phenylenediamine method (11, 12) using a pH of 5.2 and 100 μl of serum. Liver metallothionein was measured by the ^{109}Cd-binding method (13). Serum copper and zinc concentrations were measured by atomic absorption spectrophotometry (14). Statistical analyses were performed using the nonparametric Mann–Whitney–Wilcoxon test (15).

Results. The data presented in Table I demonstrate that zinc administration will induce ceruloplasmin as well as metallothionein. Adjuvant inflammation was associated with elevated serum copper concentrations. Metallothionein levels were increased twofold 2 days after adjuvant treatment. Although liver metallothionein levels decreased 6 days after adjuvant, daily zinc treatment for this period produced an eightfold increase in these levels.

13-*cis*-Retinoic acid administration via an oral route resulted in a marked induction of ceruloplasmin and a small increase in liver metallothionein (Table II). When the two treatments are compared (Tables I and II), 13-*cis*-retinoic acid appears to be a more potent inducer of ceruloplasmin than is zinc within the 6-day treatment period. Combined treatment of zinc with 13-*cis*-retinoic acid resulted in an increase in ceruloplasmin levels over those produced by either zinc or 13-*cis*-retinoic acid alone. The increase in serum ceruloplasmin caused by 13-*cis*-retinoic acid was paralleled by increases in serum copper and decreases in serum zinc.

As shown in Table III both zinc and 13-*cis*-retinoic acid reduced the acute inflammatory response following adjuvant injection. After 6 days of treatment with zinc, paw weight (edema) was decreased by about 20%. 13-*cis*-Retinoic acid reduced paw weight by 13%. Combined treatment with zinc and 13-*cis*-retinoic acid seemed to potentiate the effect of either treatment alone. It is clear the decreased paw weight was concurrent with

TABLE I. EFFECT OF ZINC ON SERUM CERULOPLASMIN, COPPER, AND ZINC, AND LIVER METALLOTHIONEIN

Group[a]	Treatment	*N*	Serum copper[b]	Serum ceruloplasmin[c]	Liver metallothionein[d]	Serum zinc[b]
Control	None	4	1.1 ± 0.1	24 ± 2	10 ± 1	1.1 ± 0.1
Adjuvant	None, 2 days	6	1.6 ± 0.1*	42 ± 2*	58 ± 11*	1.0 ± 0.05
Adjuvant	Zinc, 2 days[e]	6	1.8 ± 0.1*	52 ± 5*	111 ± 17*	1.0 ± 0.04
Adjuvant	None, 6 days	3	1.5 ± 0.2*	40 ± 3*	7 ± 1	1.2 ± 0.1
Adjuvant	Zinc, 6 days[e]	3	1.8 ± 0.1*	49 ± 2*,**	59 ± 27*,**	1.1 ± 0.1

[a] Means ± SE are shown.
[b] Concentration in μg/ml.
[c] Units/ml.
[d] μg/g liver (wet wt).
[e] Zinc at 2 mg/kg (ip) daily starting the day prior to adjuvant.
* Significantly different from control at $P < 0.05$.
** Significantly different from 6-day arthritic group at $P < 0.05$.

TABLE II. EFFECT OF 13-*cis*-RETINOIC ACID ON SERUM CERULOPLASMIN, COPPER, AND ZINC, AND LIVER METALLOTHIONEIN

Group[a]	Treatment	Serum copper[b]	Serum ceruloplasmin[c]	Liver metallothionein[d]	Serum zinc[b]
Adjuvant	None	1.4 ± 0.1	38 ± 5	10 ± 1	1.3 ± 0.1
Adjuvant	Retinoic acid[e]	2.6 ± 0.2*	83 ± 6*	20 ± 4*	1.1 ± 0.1*
Adjuvant	Retinoic acid and zinc	3.0 ± 0.3*	102 ± 11*,**	127 ± 48*,**	1.1 ± 0.04*

[a] Each group consisted of four rats; Means ± SE are shown.
[b] Concentration in μg/ml.
[c] Units/ml.
[d] μg/g liver.
[e] 13-*cis*-Retinoic acid at 160 mg/kg (po) and zinc at 2 mg/kg (ip) were given daily for 6 days starting the day prior to adjuvant.
* Significantly different from nontreated group at $P < 0.05$.
** Significantly different from retinoic acid-treated group at $P < 0.05$.

the elevation in serum ceruloplasmin levels. For example, the paw weights of rats shown in experiment 1 (Table III) correspond to the ceruloplasmin levels for these rats (6 days of treatment) as shown in Table I. Similarly, the paw weights reported for experiment 4 (Table III) correspond to ceruloplasmin levels shown in Table II. Therefore, in the data presented here, and in other experiments, the reduction in paw weight was concurrent with elevated serum ceruloplasmin levels.

Discussion. Adjuvant inflammation is characterized by pronounced edema of the adjuvant-injected limb which is maximal 5 days later. This is followed by chronic systemic inflammation and arthritis which eventually affects both limbs. Many features of adjuvant-induced arthritis are common to rheumatoid arthritis in humans, for which it serves as an animal model (16). Reasoning that rheumatoid arthritis is a proliferative disease, Brinckerhoff and co-workers tested

TABLE III. EFFECT OF ZINC AND 13-*cis*-RETINOIC ACID ON ADJUVANT INFLAMMATION

Experiment	Group[a]	*N*	Treatment	Difference in paw wt[b]	% Reduction from nontreated arthritic group
1	Control	4	None	0.097 ± 0.135	
	Adjuvant	3	None, 6 days	0.533 ± 0.007	
	Adjuvant	3	Zinc, 6 days[c]	0.424 ± 0.029*	20
2	Adjuvant	4	None, 6 days	0.778 ± 0.046	
	Adjuvant	4	Zinc, 6 days[c]	0.632 ± 0.026*	19
3	Control	4	None	0.009 ± 0.015	
	Adjuvant	3	None, 6 days	0.651 ± 0.006	
	Adjuvant	3	Retinoic acid and zinc, 6 days[c]	0.484 ± 0.034*	26
4	Adjuvant	4	None, 6 days	0.628 ± 0.067	
	Adjuvant	4	Retinoic acid, 6 days[c]	0.544 ± 0.018	13
	Adjuvant	4	Retionoic acid and zinc, 6 days[c]	0.435 ± 0.026*	31

[a] Means ± SE are shown.
[b] Difference in weight between the adjuvant-injected paw (left) and the right paw (in g).
[c] 13-*cis*-Retinoic acid at 160 mg/kg (po) and zinc at 2 mg/kg (ip) were given daily for 6 days starting the day prior to adjuvant.
* Significantly different from adjuvant nontreated group at $P < 0.05$.

the effect of 13-*cis*-retinoic acid on adjuvant inflammation and arthritis because of the effect this retinoid has on other proliferative disorders (8). They observed a reduction in paw volume of ca. 14% when the retinoid was administered daily (at 160 mg/kg) for 5 days after adjuvant. Our results correspond exactly, since we found a reduction in paw weight of 13% at this dosage of 13-*cis*-retinoic acid (Table III).

The effect of zinc administration on reduction of the initial inflammatory response to adjuvant was equally as pronounced. Simkin has shown evidence that many clinical symptoms of rheumatoid arthritis are reduced by oral therapy with zinc sulfate (9). To our knowledge, the data presented here are the first to show the effect of zinc in reducing a parameter of arthritis in an animal model. Of relevance to this finding was the observation by Kishore *et al.* (17) that paw edema in copper-deficient and copper-sufficient rats with adjuvant arthritis was inversely related to the plasma zinc concentration. This supports the results of the present study where zinc therapy reduced the same parameter. However, the results of the present study with zinc given intraperitoneally contrast with those of Lewis (18) who did not find an effect of oral zinc (50 mg/kg) in reducing kaolin-induced paw edema.

The relationship of ceruloplasmin and copper to arthritis, although it has been studied for years, is still a matter of controversy. Presumably, the effect is related to the anti-inflammatory effects of some copper complexes (18, 19) and that ceruloplasmin has functional copper atoms which may allow the protein to function as a free radical scavenger (6). In agreement with this is the observation that inflammatory responses appear to be greater in copper-deficient rats (2, 17, 21). Moreover, plasma or serum ceruloplasmin increases during arthritis along with other acute phase proteins (19) and ceruloplasmin accumulates in synovial fluid (20). The data provided by Denko (2) showing a direct effect of ceruloplasmin injections on foot swelling due to experimental inflammation in rats are extremely relevant to an anti-inflammatory role for the protein.

The mechanism that may account for increased serum ceruloplasmin in arthritic rats after either zinc or 13-*cis*-retinoic acid could be related to changes in leukocytes. Zinc has been reported to have a stabilizing effect on various leukocytes (22) and macrophages (23). As a result, these cellular sources may increase interleukin-1 levels. Interleukin-1 is known to stimulate ceruloplasmin production (24, 25) as well as metallothionein synthesis (26). In this regard, it is of relevance to mention that Dinarello (27) has proposed that treatment of many diseases which involve interleukin-1 mediation may be contraindicated because therapy interferes with normal host defense processes, including production of acute phase proteins. We have shown that 13-*cis*-retinoic acid also increases ceruloplasmin, perhaps indirectly via interleukin-1, although this is unlikely since it blocks the effect of this hormone on collagenase production in culture (28). A more likely explanation is that the induction of ceruloplasmin by 13-*cis*-retinoic acid occurs via a change in gene transcription since retinoids may have that mechanism of action (29–31). It cannot be ruled out that the increase in ceruloplasmin could be due to an inflammatory effect of the treatments themselves, however.

The induction of metallothionein in arthritic rats is also relevant to rheumatoid arthritis in humans. The protein is induced by and binds gold (32, 33). This induction could have a negative effect on the anti-inflammatory action of gold salts used for treatment of arthritis. Furthermore, it suggests that a therapeutic regimen that leads to increased metallothionein production, e.g., gold, zinc, or glucocorticoids may be counterproductive, whereas 13-*cis*-retinoic acid and similar agents which have little effect on metallothionein may be useful as combination or add-on therapies for anti-inflammatory treatments with agents that induce the protein.

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