Effects of Growth, Food Intake, and Dietary Zinc on Diadenosine Tetraphosphate Concentrations in Rats

John K. Chesters, Ronald Boyne, and Linda Petrie

Rowett Research Institute, Bucksburn, Aberdeen, UK

The nucleotide diadenosine tetraphosphate has been suggested to function as a signal molecule for the initiation of DNA replication. Previous studies have indicated that diadenosine tetraphosphate is synthesized by certain aminoacyl tRNA synthetases and that diversion of AMP from the amino acid-enzyme complex to ATP to form diadenosine tetraphosphate is facilitated by zinc ions. The growth retardation of zinc-deficient rats is associated with specific reduction in DNA replication and also with a potentially growth-limiting decrease in food intake. The possibility has been investigated that in zinc-deficient rats, lack of Zn^{2+} restricts diadenosine tetraphosphate synthesis, resulting in a failure to synthesize DNA and in a reduction in growth. The results indicate that the depressed growth potential caused by the reduction in food intake associated with the deficiency was sufficient to lower diadenosine tetraphosphate concentrations significantly in the liver and spleen. However, there was no indication of a specific effect of zinc deficiency on diadenosine tetraphosphate values.

Keywords: zinc; growth; diadenosine tetraphosphate; food intake.

Introduction

The dinucleotide 5',5'-diadenosine tetraphosphate (DAT) has been suggested to act as a secondary messenger serving as an intermediate signal molecule between membrane events resulting from mitogenic stimulation and the subsequent initiation of nuclear DNA synthesis.[1] Intracellular concentrations of DAT have been shown to increase markedly in correlation with, and generally slightly in advance of, the increase in DNA synthesis in the liver of partially hepatectomized rats[2] and in cultures of quiescent BHK and 3T3 cells stimulated with serum.[3] Furthermore, addition of DAT to permeabilized BHK cells arrested in the G1 phase of growth stimulated the cells to synthesize DNA.[4]

All currently recognized methods for the biologic synthesis of DAT involve modified activity of certain aminoacyl tRNA synthetases, resulting in the transfer of the AMP moiety from an amino acid-AMP-synthetase complex to a molecule of ATP to form DAT. This reaction has been observed in both bacterial[5] and eukaryotic systems,[6] and in each case, synthesis of DAT was markedly enhanced by the addition of zinc ions (Zn^{2+}). Lack of dietary zinc has been shown to inhibit growth and, in particular, to impair DNA synthesis in animals; this seems to result from a reduced ability to initiate the events leading to DNA synthesis rather than from an impairment of its synthesis *per se*.[7] At least some of the biologic effects of dietary zinc deficiency could thus be explained by a need for Zn^{2+} during the synthesis of DAT if this compound then acted as a second messenger for the initiation of DNA replication.

On the other hand, the concentrations of Zn^{2+} used in studies of DAT synthesis *in vitro* have ranged from low micromolar to near millimolar in the presence of a varied assortment of compounds capable of binding Zn^{2+}.[5-9] Current estimates of the concentration of "free" Zn^{2+} in mammalian systems are of the order of one nanomolar or less[7]; thus, the relevance of the effects of Zn^{2+} ions on DAT synthesis *in vitro* to the biologic role of zinc in mediating DNA synthesis in animals is uncertain. However, Grummt et al.[1] showed that treatment of BHK cells with EDTA inhibited both DNA and DAT synthesis to comparable extents and that the effects of EDTA were reversed specifically by the addition of Zn^{2+} ions. Furthermore, the concentrations of free Zn^{2+} likely to have been present under the

Address reprint requests to Dr. J. K. Chesters, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, UK.
Received September 29, 1989; accepted October 13, 1989.

experimental conditions used were comparable to those present *in vivo*.

The possibility that reduced DAT synthesis underlies the impaired growth of zinc-deficient rats has been investigated. However, when rats are rendered zinc-deficient by feeding a diet low in zinc, the reduction in their growth is accompanied by a corresponding decrease in voluntary food intake sufficient to prevent the growth of pair-fed rats restricted to equivalent amounts of zinc-supplemented diet.[10] Furthermore, the reduction in food intake is not uniform, but varies markedly from day to day both within and between rats. Care has therefore been taken in the present experiments to distinguish effects on DAT synthesis associated with reduced zinc availability from those induced by low and variable food intake.

Methods and Materials

Animals

Male hooded Lister rats of the Rowett Institute strain weighing approximately 80 g were housed in perspex and glass cages and offered a zinc-adequate semisynthetic diet for 1 week.[11] They were then randomly allocated to groups of eight and subjected to one of the following dietary regimens designed to provide adequate control for changes in DAT content induced merely by the low and cyclic food intake of zinc-deficient rats.[10] In the first regimen, one group of rats was offered a zinc-deficient diet (<0.5 mg Zn/kg) *ad libitum* for only 4 days while another group continued on zinc-adequate diet (40 mg Zn/kg) *ad libitum* for the same period. In an alternative strategy, groups of rats were offered the zinc-deficient diet *ad libitum* for 12 days, by which time they were clearly zinc-deficient as indicated by their growth having stopped and by their characteristic cyclic pattern of food intake. The rats were then transferred to a fixed cycle of food intake which consisted of successive daily quantities of zinc-deficient diet of 14, 5, and 2 g. This cycle, which was repeated, approximated the natural pattern of food intake of zinc-deficient rats of this size and was used to standardize the quantities of food eaten on the day of study.[12] Certain rats were investigated at the end of the seventh day on the imposed cycle after a 14-g allocation of food, these rats being classed as −Zn high intake. As a control, the rats of another group were offered the zinc-adequate diet throughout, but were individually pair-fed with the deficient rats for the first period then transferred to the same fixed cycle of intake for the final 7 days. Finally, the rats of the −Zn low intake group had the pattern of the imposed food cycle shifted by 1 day to give a final intake of 2 g. Rats of the corresponding +Zn low intake group were pair-fed with zinc-deficient rats as above and also finished with a low food intake of 2 g. Each experiment consisted of a zinc-deficient group from one of the above regimens and its corresponding control group.

Preparation of tissue extracts

At the end of the experimental period, the rats were killed by a blow on the head, and the liver, heart, and spleen were removed. The tissues were immediately frozen in liquid nitrogen and stored at −20°C until required. They were then powdered while still frozen, and approximately 1.2 g of the powder from individual livers and pooled pairs of spleens or hearts was homogenized in twice its weight of ice-cold 0.7 M perchloric acid using an Ultra-Turrax blender (Janke & Kunkel Staufen, West Germany). Two identical samples of each homogenate were then processed after the addition of a known amount of DAT to one sample to act as a recovery standard. After 20 minutes on ice, the samples were centrifuged, and the supernatant was removed and neutralized with potassium hydroxide. The preparations were heated to 100°C for 5 minutes to destroy endogenous phosphodiesterase activity, then cooled in ice for 20 minutes. Insoluble potassium perchlorate was removed by centrifugation and the supernatant was stored at −20°C until it was used for DAT assay.

Assay of diadenosine tetraphosphate

Aliquots of the supernatants (0.5 ml each) were added to 10 ml of 0.12M ammonium carbonate buffer, pH 8.2, and the mixture was passed through a 0.4 ml DEAE-Sephacel column. The column was washed with a further 5 ml of 0.12M buffer to remove the majority of the ATP, and DAT was recovered by elution with 2 ml of 0.3M ammonium carbonate buffer, pH 8.2. One milliliter of the eluate was treated with 50 U of calf intestinal alkaline phosphatase (BCL, London, England) at 37°C for 30 minutes to destroy residual ATP and the enzyme was then inactivated by heating at 100°C for 2 minutes before being cooled to room temperature and adjusted to pH 8.2. The preparations were then centrifuged to remove denatured protein and used for DAT assay.

Diadenosine triphosphate was assayed by the addition of 50 μl of eluate to 150 μl 10 mM tris-HCl buffer, pH 8.0, and 50 μl ATP monitoring reagent (LKB, Milton Keynes).[13] The reaction was started by the addition of 20 μl phosphodiesterase 1 (Sigma Chemical Co., Poole, Dorset) and the count rate was determined 12 minutes later using a Model 1250 luminometer (LKB, Miton Keynes). The count rate was shown to be proportional to DAT concentration under these conditions, and each sample was calibrated by the measurement of a duplicate sample to which a known amount of DAT (IS) had been added. The basal count rate associated with any residual ATP was also estimated in another replicate without added phosphodiesterase.

Diadenosine triphosphate concentrations were calculated from the luminometer count rates using the following relationship:

$$DAT\ concentration = \frac{(Exptal - ATP\ Blank)}{(IS - Exptal)} \times IS\ DAT\ concentration$$

The concentration of DAT in the wet weight of the original tissue sample was then calculated from the

relevant dilution factors. This was then corrected for the recovery (generally 60% to 70%) of the standard amount of DAT initially added to the second sample of the original homogenate which was processed in parallel with the first.

Results

Initial investigations indicated that DAT was unstable in 0.7 M perchloric acid extracts of liver following removal of the perchloric acid by neutralization with potassium hydroxide. The activity that destroyed the DAT, possibly an endogenous acid-stable phosphodiesterase, was found to be heat labile and advantage was taken of the relative stability of DAT to heat to stabilize the extracts by brief heating at 100°C. The accuracy and sensitivity of the final assay of DAT crucially depended on reducing contaminating ATP concentrations to very low levels. Use of small DEAE-Sephacel columns permitted removal of most of the ATP without excessive dilution of the sample, but unacceptably high ATP concentrations still remained. However, subsequent treatment with alkaline phosphatase destroyed most of the residual ATP without affecting the DAT, and the phosphatase was then totally inactivated by heating before the DAT was assayed. Any losses of DAT incurred during heating and processing were compensated for by correction of the experimental values for the recovery of the standard DAT added to the duplicate sample.

The results presented in *Table 1* indicate marked trends toward a reduction in liver weight and DAT concentration per gram wet weight in the zinc-supplemented rats associated with restriction of food intake during the 24 hours immediately preceding their death. Analysis of the data indicate that both parameters were highly significantly correlated ($P < 0.001$) with food intake. This dual correlation was associated with an even more marked reduction in total DAT contents of the livers of the zinc-adequate rats as their food intake was restricted. In contrast, DAT concentrations in the livers of the zinc-deficient rats were relatively little affected by variations in food intake. After only 4 days on the zinc-deficient diet, before food intake and growth are normally affected by the deficiency, there was a marked reduction in the DAT content of the liver of the zinc-deficient rats. However, in this particular experiment, the onset of the deficiency was so rapid that both growth and food intake were also greatly reduced at this time. Viewed overall, once allowance was made for the effects of food intake on the control animals, the concentrations of DAT in the livers of the zinc-deficient rats tended to be higher than those in the control animals rather than lower.

The heart showed no discernible trend in either weight or DAT concentration with zinc status or food intake. In contrast, the weights of the spleens of the zinc-adequate rats correlated ($P < 0.001$) with the animal's food intake during the previous 24 hours. With each pattern of food intake, the concentration of DAT in the spleens of the zinc-deficient rats was actually higher than that in the controls rather than lower, as would have been expected if the deficiency impaired the synthesis of DAT. However, in no case was the difference statistically significant. Furthermore, the similarity between the values for zinc-deficient and control spleens was even closer when the total DAT contents of the spleens were compared between groups.

Discussion

It is clear from the results obtained with liver and spleen that changes in food intake in the zinc-adequate rats were associated with at least as large reductions in

Table 1 Influence of food intake and dietary zinc concentration on the wet weights and diadenosine tetraphosphate concentrations of the liver, heart, and spleen of rats

		Body weight (g)	Food intake (g)	Liver		Heart		Spleen	
				Weight (g)	DAT (pmol/g)	Weight (g)	DAT (pmol/g)	Weight (g)	DAT (pmol/g)
4-day deficient									
−Zn	Mean	140.5^{a}	5.4^{a}	5.69^{a}	1065^{ab}	0.60^{ab}	550^{a}	0.54^{a}	1059^{a}
	SEM	3.4	1.0	0.31	120	0.03	30	0.03	182
+Zn	Mean	157.1^{bc}	16.0^{b}	8.57^{b}	1601^{b}	0.65^{b}	360^{ab}	0.70^{b}	652^{bc}
	SEM	2.7	0.4	0.26	238	0.01	93	0.04	91
High food intake									
−Zn	Mean	135.1^{a}	9.5^{c}	5.82^{a}	1096^{ab}	0.51^{c}	277^{b}	0.43^{c}	756^{ab}
	SEM	3.2	0.7	0.18	203	0.01	35	0.01	34
+Zn	Mean	167.7^{bc}	14.0^{d}	6.89^{c}	1241^{ab}	0.56^{ac}	265^{b}	0.51^{ac}	682^{abc}
	SEM	3.2	0.0	0.12	237	0.01	20	0.01	58
Low food intake									
−Zn	Mean	136.3^{a}	2.0^{e}	4.76^{d}	853	0.52^{c}	319^{bc}	0.37^{c}	377^{bc}
	SEM	5.3	0.0	0.22	103	0.01	12	0.01	82
+Zn	Mean	146.4^{bc}	2.0^{e}	4.80^{d}	761	0.55^{ac}	523^{ac}	0.44^{ac}	288^{c}
	SEM	4.2	0.0	0.12	64	0.02	83	0.03	31

Values are mean and SEM values for groups of eight rats, but the DAT concentrations in the spleen and heart were estimated using combined pairs of these organs.
Statistical Analysis.
Statistical significances were assessed by analysis of variance.
Within a column, values without a common superscript were significantly different ($P < 0.05$).

DAT concentration as those produced in the same tissues of rats fed a zinc-deficient diet. Furthermore, in both the liver and spleen of the rats fed the zinc-deficient diet for the longer period, a low food intake in the final 24 hours was associated with a tendency to reduced DAT concentration compared with those after a final high intake. Such a low food intake in rats has been shown previously to be associated with relatively elevated plasma zinc concentrations[14] and with prevention of the fetal abnormalities frequently induced by zinc deficiency during pregnancy.[15] Thus, low food intake by zinc-deficient rats is generally associated with a slight increase rather than decrease in zinc availability and is probably caused by tissue catabolism following low food intake. Such an increased availability of zinc might have been expected to increase DAT concentrations, whereas it was actually found to be associated with a reduction in DAT similar to that caused by restricted food intake in the zinc-adequate control animals. The effects on DAT metabolism in the zinc-deficient rats can therefore be readily explained as being secondary to changes in food intake which appear to have been a more powerful determinant of DAT concentration than the adequacy of the zinc supply. The fact that food intake had a greater influence on DAT concentrations in the zinc-adequate than in the zinc-deficient rats possibly indicates that DAT concentrations were more closely related to growth potential than to food intake *per se*. Thus, the day-to-day growth of the zinc-adequate animals varied markedly with their food intake, and this was reflected in their tissue DAT concentrations. In contrast, in the zinc-deficient rats, in which growth was prevented at all times by a lack of zinc, similar variations in food intake produced relatively little effect on DAT concentrations.

In conclusion, the present results support the view[1,16] that DAT concentrations are related to growth potential, but do not suggest that the restriction of growth which accompanies zinc deficiency in rats is mediated by an inability to synthesize DAT.

References

1 Grummt, F., Weinmann-Dorsch, C., Schneider-Schaulies, J., and Lux, A. (1986). Zinc as a second messenger of mitogenic induction. *Exp. Cell Res.* **163,** 191–200

2 Yamaguchi, N., Kodama, M., and Ueda, K. (1983). Diadenosine tetraphosphate as a signal molecule linked with the functional state of rat liver. *Gastroenterology* **89,** 723–731

3 Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F. J., Fries, R. R., Pierron, G., Moll, W., and Grummt, F. (1984). Drastic rise in intracellular adenosine 5′-tetraphosphate 5′adenosine correlates with onset of DNA synthesis in eukaryotic cells. *Eur. J Biochem.* **138,** 179–185

4 Rapaport, E., and Zamecnik, P. C. (1976). Presence of diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) in mammalian cells in levels varing widely with proliferative activity of the tissue: a possible positive "pleiotypic activator." *Proc. Natl. Acad. Sci. USA* **73,** 3984–3988

5 Goerlich, O., and Holler, E. (1984). Phenylalanyl-tRNA synthetase of E. coli K10. Effects of Zn(II) on partial reactions of diadenosine 5′,5‴-P1,P4 tetraphosphate synthesis, conformation and protein aggregation. *Biochemistry* **23,** 182–190

6 Brevet, A., Plateau, P., Cirokoglu, B., Palliez, J.-P., and Blanquet, S. (1982). Zn-dependent synthesis of 5′,5′-diadenosine tetraphosphate by sheep liver lysyl- and phenylalanyl-tRNA synthetases. *J. Biol. Chem.* **257,** 14613–14615

7 Chesters, J. K., and Arthur, J. R. (1988). Early biochemical defects caused by dietary trace element deficiencies. *Nutr. Res. Rev.* **1,** 39–56

8 Blanquet, S., Plateau, P., and Brevet, A. (1983). The role of Zn in 5′,5′diadenosine tetraphosphate production by aminoacyl-tRNA synthetases. *Mol. Cell. Biochem.* **52,** 3–11

9 Zamecnik, P. C. (1983). Diadenosine 5′5‴P1,P4 tetraphosphate (Ap4A): its role in cellular metabolism. *Anal. Biochem.* **134,** 1–10

10 Chesters, J. K., and Quarterman, J. (1970). Effects of zinc deficiency on food intake and feeding patterns of rats. *Br. J. Nutr.* **24,** 1061–1069

11 Davies, N. T., and Reid, H. (1979). An evaluation of the phytate zinc copper iron and manganese contents and Zn availability from soya-based textured-vegetable-protein meat substitutes and meat replacers. *Br. J. Nutr.* **41,** 579–589

12 Record, I. R., Dreosti, I. E., Tulsi, R. S., and Manuel, S. J. (1986). Maternal metabolism and teratogenicity in Zn-deficient rats. *Teratology* **33,** 311–317

13 Ogilivie, A. (1981). Determination of diadenosine tetraphosphate (Ap_4A) levels in subpicomole quantities by a phosphodiesterase luciferin-lucerferase coupled assay; application as a specific assay for diadenosine tetraphosphatase. *Anal. Biochem.* **115,** 302–307

14 Chesters, J. K., and Will, M. (1973). Some factors controlling food intake by zinc-deficient rats. *Br. J. Nutr.* **30,** 555–566

15 Dreosti, I. E., Record, I., and Manuel, S. J. (1985). Zinc deficiency and the developing embryo. *Biol. Trace Element Res.* **7,** 103–122

16 Baril, E. P., Coughlin, S. A., and Zamecnik, P. C. (1985). 5′5‴P1P4 diadenosine tetraphosphate (Ap4A): a putative initiator of DNA replication. *Cancer Invest.* **3,** 465–471