Zinc, vanadate and selenate inhibit the tri-iodothyronine-induced expression of fatty acid synthase and malic enzyme in chick-embryo hepatocytes in culture

Yuan ZHU,* Alan G. GOODRIDGE† and Susan R. STAPLETON*‡

*Department of Chemistry, Western Michigan University, Kalamazoo, MI 49008, U.S.A., and †Department of Biochemistry, University of Iowa, Iowa City, IA 52242, U.S.A.

Insulin regulates the expression of genes involved in a variety of metabolic processes. In chick-embryo hepatocytes in culture, insulin amplifies the tri-iodothyronine (T_3)-induced enzyme activity, and the level and rate of transcription of mRNA for both fatty acid synthase (FAS) and malic enzyme (ME). Insulin alone, however, has little or no effect on the expression of these genes. In chick-embryo hepatocytes, the mechanism by which insulin regulates the expression of these or other genes is not known. Several recent studies have compared the effects of zinc, vanadate and selenate on insulin-sensitive processes in an attempt to probe the mechanism of insulin action. Because zinc, vanadate and selenate mimic the effects of insulin on several processes, they have been termed insulin-mimetics. We have studied the effect of zinc, vanadate and selenate on the T_3-induced expression of both FAS and ME. Like insulin, these agents had little or no effect on the basal activities for FAS and ME in chick-embryo hepatocytes in culture for 48 h. Unlike insulin, however, zinc, vanadate and selenate inhibited the T_3-induced activities and mRNA levels of both FAS and ME. Maximal inhibition was achieved at concentrations of 50 μM zinc or vanadate, or 20 μM selenate. Zinc and vanadate also inhibited the T_3-induced transcription of the FAS and ME genes. Although the mechanism of this inhibition is unknown, our results indicate that it is not mediated through inhibition of binding of T_3 to its nuclear receptor nor through a general toxic effect. Thus zinc, vanadate and selenate are not insulin-mimetics under all conditions, and their effects on other insulin-sensitive processes may be fortuitous and unrelated to actions or components of the insulin signalling pathway.

INTRODUCTION

Insulin regulates the expression of many genes involved in a variety of metabolic processes. The mechanism by which insulin regulates gene expression is not known. In fact, this hormone may regulate different genes by different mechanisms. In a number of recent studies, zinc, vanadate and selenate have been found to mimic the action of insulin on several processes. The results from these types of studies has generated enthusiasm in using these mimetics to try to define the mechanisms of insulin action. In streptozotocin-induced diabetic rats, the activities of hepatic malic enzyme (ME) and glucose-6-phosphate dehydrogenase are very low. Oral administration of vanadate to these animals causes the activities to return to the normal range [1]. Hexokinase, pyruvate kinase [2] and enzymes involved in glycogenolysis [3] are similarly affected by diabetes and vanadate. Marked improvement in glucose homoeostasis also has been observed in diabetic animals treated with vanadate [4–6]. In chicken liver, both vanadate and insulin inhibit fructose-2,6-bisphosphatase activity and gluconeogenesis [7]. In a variety of cell types in culture, vanadate modulates arachidonic acid metabolism [8], inhibits expression of transfected phosphoenolpyruvate carboxykinase genes [9], stimulates mRNA accumulation and transcription of gene 33 [10], and causes phosphorylation of potential target proteins of the insulin receptor kinase [11]; again, all effects similar to those of insulin. Selenate [12,13], zinc [14] and chromate [15] also regulate glucose uptake and other metabolic processes in a manner similar to that of insulin. Clearly, the data that have been reported to date show that vanadate, selenate, chromate and zinc have the same effects on many metabolic processes as insulin and that these effects occur in a variety of test systems.

In chick-embryo hepatocytes in culture, insulin significantly amplifies the tri-iodothyronine (T_3)-induced enzyme activity, and the level and rate of transcription of mRNA for both fatty acid synthase (FAS) and ME [16,17], but has little or no effect by itself. In an effort to understand the mechanism of action of insulin in this system, we tested the effects of vanadate, selenate, chromate and zinc on the induction of ME and FAS gene expression caused by T_3. Surprisingly, vanadate, selenate and zinc inhibited the effects of T_3 and chromate had no effect.

EXPERIMENTAL

Materials

Crystalline bovine insulin was a gift from Eli Lilly Corp. Proteinase K was purchased from Boehringer Mannheim Biochemicals. SP6-grade [^{32}P]UTP (800 Ci/mmol) and [^{32}P]dCTP (800 Ci/mmol) were purchased from Amersham Corp. and ICN Biochemicals respectively. [^{125}I]T_3 (150 μCi/μg) was purchased from DuPont/NEN. Nucleotides used in the transcription assays were from Pharmacia. All other enzymes or hormones not specifically indicated in the text were obtained from Sigma Chemical Corp.

Preparation and maintenance of isolated cells

Unincubated embryonated eggs from white Leghorn chickens were obtained from Hy Vac Laboratory Eggs, Gowrie, IA, or Townline Poultry, Zeeland, MI, U.S.A., and incubated in an

Abbreviations used: FAS, fatty acid synthase; ME, malic enzyme; ICDH, isocitrate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; T_3, tri-iodothyronine.

‡ To whom correspondence should be addressed.

electric forced-draught incubator at 37.5±0.5 °C and 60% relative humidity. Embryos (18 or 19 days of incubation) were killed by decapitation. Liver cells were isolated [18] and suspended in serum-free Waymouth MD705/1 medium (Gibco Laboratories, Life Technologies Inc.) containing penicillin (60 μg/ml) and streptomycin (100 μg/ml) and placed in untreated Petri dishes of diameter either 90 mm or 60 mm for transcription assays or enzyme assays and RNA isolations, respectively. The cells were then incubated at 40 °C in an atmosphere of air/CO_2 (19:1). Serum-free Waymouth medium (9 ml) was incubated with 1 ml of the cell suspension (2–3 mg of total protein, about 1×10^7 cells). After about 20 h of incubation, the medium was changed and hormones were added as indicated in the Figure legends. ME activity [19], FAS activity [20] and protein [21] were assayed by the indicated methods. Isocitrate dehydrogenase (ICDH) activity was determined by substituting 5 mM isocitrate for malate in the ME assay. ATP levels were measured with the firefly luciferase assay [22]. Lactate dehydrogenase was assayed as previously described [23].

DNA probes

The cDNA probes for chicken FAS were generously provided by Dr. Gordon Hammes [24]. FAS 1 and FAS 2 correspond to bases 1–2813 and 2814–4180, respectively of a cDNA corresponding to the 3′ end of FAS mRNA. FAS 1 includes sequences from the 3′ coding region, and FAS 2 includes sequences from the 3′ untranslated region. Cloned genomic DNAs and cDNAs for avian ME have been described [17]. ME-2.3 contains both intronic and exonic sequences from the 3′ end of the gene. The cDNA probes for chicken β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were gifts from Dr. Don Cleveland [25] and Dr. R. J. Schwartz [26] respectively.

Isolation and measurement of mRNA levels

After removing the medium, RNA was isolated from the cells by the guanidinium thiocyanate/phenol/chloroform method [27]. Total RNA was treated with formaldehyde and subjected to electrophoresis in 1.0% agarose gels [28]. The separated RNAs were transferred to GeneScreen (NEN/DuPont) and hybridized with labelled cDNAs for FAS, ME and GAPDH. The cDNA was labelled by the random-primer method (Amersham Kit) according to the manufacturer's instructions. The membranes were hybridized, washed [29] and subjected to autoradiography at −70 °C with Kodak XAR-5 film and intensifying screens. The exposed films were quantified by densitometry.

Isolation of nuclei and transcription run-on assay

Isolation of the nuclei from chick-embryo hepatocytes in culture was previously described [16,30]. The isolated nuclei were resuspended in 50% glycerol/50 mM Hepes (pH 7.4)/150 mM NaCl/0.1 mM EDTA/10 mM dithiothreitol/0.25 mM phenylmethanesulphonyl fluoride and stored at −70 °C until used.

The assay of transcription *in vitro* was carried out as described [31,32]. A 100 μl portion of resuspended nuclei was used in the assay. The reaction was incubated at 26 °C for 20 min and terminated by addition of 11 μg of RNAase-free DNAase I (BRL). After 10 min at room temperature, SDS, EDTA, yeast tRNA and proteinase K were added to final concentrations of 0.2%, 1 mM, 25 μg and 50 μg respectively. The incubation was continued at 37 °C for an additional 1.5 h. The solution was extracted twice with phenol/chloroform (1:1, v/v) and once with 1 vol. of chloroform. The labelled transcripts were precipitated with ethanol, and final purification was achieved by passing the resuspended transcripts over a 'Nick' column (Pharmacia).

The labelled transcripts [$(20–30) \times 10^6$ c.p.m.] were added to prehybridized filters slotted with 2 μg of the specific DNAs and hybridized for 72 h at 65 °C. The filters were washed sequentially in 2 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate) (4 × 5 min) at room temperature, 2 × SSC + 10 μg/ml RNAase A (1 × 15 min) at 37 °C, 0.3 × SSC/1% SDS (1 × 15 min) at 65 °C, and 0.3 × SSC (1 × 15 min) at room temperature. Autoradiography and densitometric scanning were as described above.

T_3-binding assay

The effect of vanadate, zinc and selenate on the binding of [^{125}I]T_3 to T_3 receptors was determined. Isolated hepatocytes were prepared as described above and incubated in medium for approx. 20 h. The medium was changed to one containing either no other additions or a mimetic and incubated for an additional 24 h. At 2 h before isolation of nuclei, [^{125}I]T_3 (200 pM) was added to the cells. Non-specific binding was determined by adding 1 μM unlabelled T_3 in addition to the [^{125}I]T_3. The nuclei were isolated as described above, washed [33] and assayed for radioactivity [34] and protein.

RESULTS AND DISCUSSION

In chick-embryo hepatocytes in culture, insulin amplifies the T_3-induced increases in both FAS and ME at the level of enzyme activity, mRNA abundance and rate of transcription [16,17]. The mechanism by which insulin causes this amplification is unknown. Several recent studies have examined the effects of zinc, vanadate, chromate and selenate on several insulin-stimulated metabolic processes [1,2,3,12,14,15]. In no instance, however, have these compounds been tested on insulin's effect on another hormone. Addition of vanadate, zinc, selenate or chromate to chick-embryo hepatocytes in culture for 48 h had little or no effect on the basal activity of FAS and ME. Unlike insulin, which augments the T_3 induction of FAS and ME, vanadate, zinc and selenate inhibited the T_3 effect. This inhibition of activity was found to be concentration-dependent (Figures 1a, 1c and 1d) [35,36]. Maximal inhibition was achieved at concentrations of 50 μM zinc or vanadate, and 20 μM selenate (Figures 1a, 1c and 1d). Addition of maximally effective concentrations of vanadate, selenate or zinc inhibited the T_3-induced increases in FAS and ME activities by at least 75% (Figure 1). The effects of vanadate, zinc and selenate were selective, because ICDH activity was unaffected (Figures 1a, 1c and 1d). At any concentration tested, chromate had no effect on the T_3-induced accumulation of FAS and ME (Figure 1b). If, as previously suggested [15], chromate exerted its effects on metabolic processes in a fashion similar to insulin, or the proposed mimetics, zinc, vanadate or selenate, then these results would be surprising. Our data, however, indicate potential mechanistic diversity among these agents.

The effects of these agents on mRNA abundance also were tested. Vanadate, zinc and selenate inhibited the T_3-induced accumulation of the mRNAs for both FAS and ME in a concentration-dependent manner (Figure 2). The percentage inhibition observed is similar to that of the enzyme activity, based on densitometric scans of the specific mRNAs relative to the control, GAPDH mRNA, which was unaffected under all conditions tested (Figure 2).

The T_3-induced increases in both FAS and ME activities and their mRNAs were correlated with comparable increases in rates

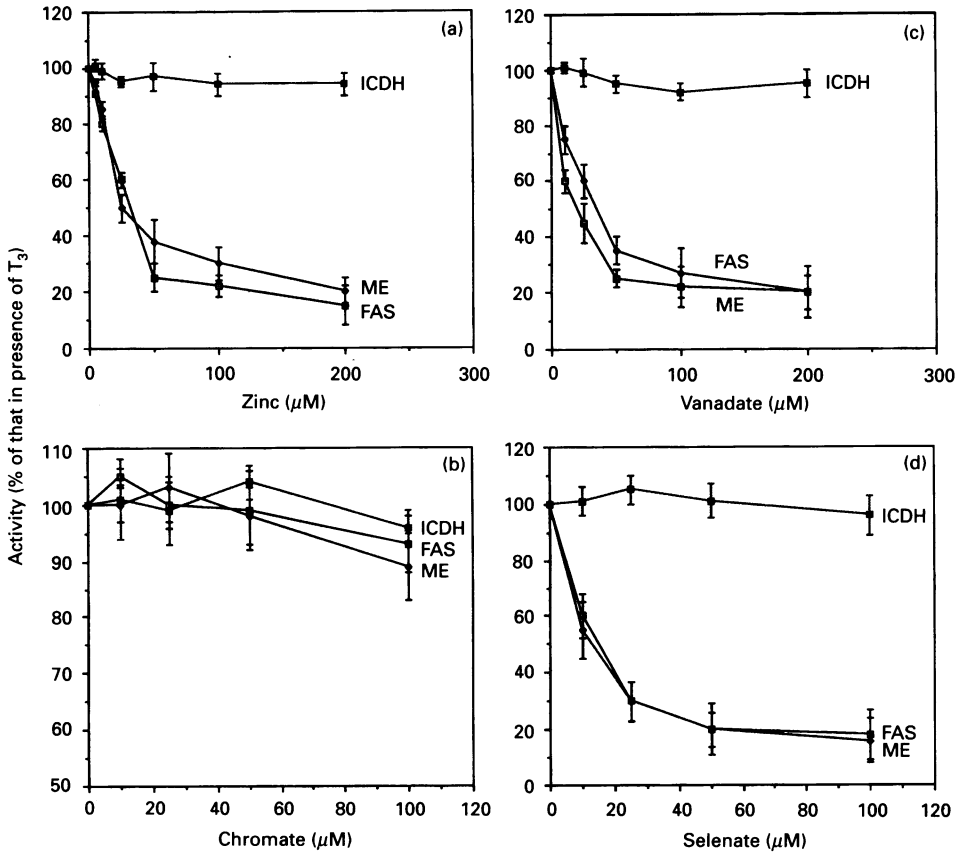

Figure 1 Concentration-dependent effects of zinc, chromium, vanadate and selenate on the T_3-induced increases in FAS and ME activities

Hepatocytes were incubated for 48 h in the presence of 1.6 μM T_3 and (**a**) 0, 5, 10, 25, 50, 100 or 200 μM zinc chloride, or (**b**) 10, 25, 50 or 100 μM ammonium chromate, or (**c**) 10, 25, 50, 100 or 200 μM sodium vanadate, or (**d**) 10, 25, 50 or 100 μM sodium selenate. The cells were harvested and the activities of FAS, ME and ICDH were determined. The results are expressed as a percentage of the activities (m-units/mg of soluble protein) for cells incubated with T_3, as means $\pm$ S.E.M ($n = 4$). In these cells in culture, T_3 typically stimulates basal activities of FAS and ME 4- and 20-fold respectively.

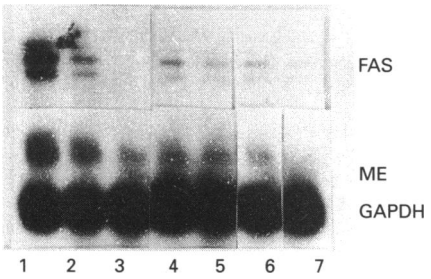

Figure 2 Concentration-dependent effects of zinc, vanadate and selenate on the T_3-induced increases in FAS and ME mRNAs

Hepatocytes were incubated for 24 h in the presence of 1.6 μM T_3 (lane 1) and zinc chloride [10 (lane 2) or 50 (lane 3) μM], or sodium vanadate [20 (lane 4) or 50 (lane 5) μM], or sodium selenate [10 (lane 6) or 50 (lane 7) μM]. Total RNA was isolated and separated by size with agarose-gel electrophoresis. The RNA was transferred to 'GeneScreen' and hybridized to labelled cDNAs for FAS, ME and GAPDH.

of transcription of their respective genes, indicating that regulation is primarily at that step [16,17]. Zinc and vanadate also were tested for their abilities to inhibit the T_3-induced rate of transcription for both FAS and ME. Both agents almost completely inhibited the T_3-induced transcription of the genes for FAS and ME (Figure 3). This inhibition was time-dependent (results not shown). Neither agent affected the transcription rate for the β-actin gene (Figure 3), nor was transcription affected if the agent was added to the assay mixture. Previously, zinc added to chick-embryo hepatocytes in culture was also shown to induce the metallothionein gene [37]. All these results are indicative of the specificity of inhibition.

The effects of these agents on T_3-induced activity, mRNA abundance and transcription of FAS and ME were the same whether insulin was present in the medium or not. Insulin did not reverse this inhibition of the T_3 induction, suggesting that vanadate, selenate and zinc are not acting through an insulin signalling pathway in these hepatocytes in culture.

By what mechanism could these agents be inhibiting T_3-induced transcription of both FAS and ME genes? The DNA-binding domain of the T_3 receptor contains a cysteine/histidine-rich region similar to that of so-called zinc-finger structures involved in binding of the protein to DNA [38,39]. Zinc is reported to inhibit release of thyroid-hormone receptor from the chromatin of GC cells in culture [40]. If zinc or any other metal were acting in this fashion, T_3-induced transcription would not be inhibited. In our studies, this was not the case. Another group has reported that zinc, and other bivalent cations such as cobalt,

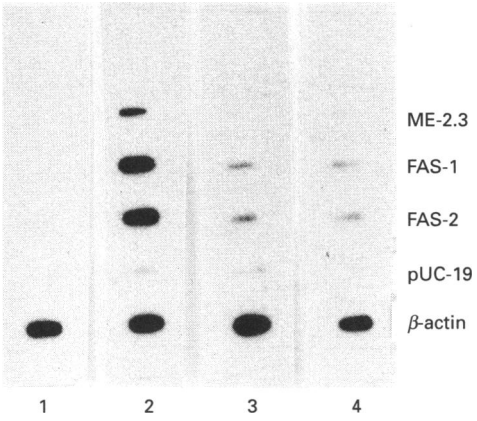

Figure 3 Effect of zinc and vanadate on the T_3-induced increases in transcription rates of the genes for FAS and ME

Hepatocytes were incubated for 24 h with either no additions (1), or 1.6 μM T_3 alone (2), or T_3 plus 50 μM zinc chloride (3), or T_3 plus 50 μM sodium vanadate (4). Nuclei were isolated and used in a transcription run-on assay as described in the Experimental section. The labelled transcripts were hybridized to slot-blots containing 2 μg of each of the following DNAs: genomic DNA for ME (ME-2.3), FAS cDNA (FAS-1 and -2), vector DNA (pUC-19) and β-actin cDNA (β-actin).

copper, nickel and manganese, inhibit binding of T_3 to its nuclear receptor in GC cells in culture [41]. None of the agents at maximal concentrations used in the present study inhibited binding of T_3 to its receptor (results not shown). This suggests that zinc, vanadate and selenate do not exert their inhibitory effects on transcription by inhibiting T_3 binding to its receptor. Inhibition of the T_3-induced transcription of FAS and ME does not appear to be due to a toxic effect caused by vanadate, selenate or zinc. Metabolic state was assessed by monitoring ATP levels and lactate dehydrogenase release into the media. Neither T_3 nor these inhibitory agents had statistically significant effects on the internal concentration of ATP or on the amount of lactate dehydrogenase released into the media (results not shown). Neither ICDH activity nor the basal activities for both FAS and ME were affected by zinc, vanadate or selenate (Figure 1), indicating no apparent general toxic effect by these agents.

These results indicate that vanadate, selenate, zinc and chromate do not mimic insulin with regard to its ability to amplify T_3-induced transcription of the FAS and ME genes in chick-embryo hepatocytes in culture. Insulin did not reverse the inhibitory actions of vanadate, selenate or zinc, suggesting that insulin and these agents act via separate mechanisms. Our results raise the possibility that the observed effects of zinc, vanadate and selenate on glucose oxidation and hexose transport may be similar to those caused by insulin, but do so by a mechanism distinct from that of insulin.

Received 13 September 1993/25 April 1994; accepted 20 May 1994

This study was supported in part by a National Institutes of Health Grant number DK43917-01A1.

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