Apolipoprotein A-I gene expression is regulated by cellular zinc status in Hep G2 cells

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Wu, John Y. J., Yan Wu, Scott K. Reaves, Yi Ran Wang, Polin P. Lei, and Kai Y. Lei. Apolipoprotein A-I gene expression is regulated by cellular zinc status in Hep G2 cells. Am. J. Physiol. 277 (Cell Physiol. 46): C537–C544, 1999.—The influence of Zn on the expression of the apolipoprotein A-I (apoA-I) gene in Hep G2 cells was examined. Zn depletion was achieved with a low-Zn (ZD) medium prepared from Zn-free growth medium (Opti), a ZD medium containing Chelex 100-extracted fetal bovine serum (CHE), and a medium containing chelator 1,10-phenanthroline (OP). Compared with those for their respective controls, cellular Zn levels were reduced by 55, 48, and 46% and apoA-I mRNA abundances were reduced by 20, 29, and 28% in Opti, CHE, and OP systems, respectively, after one passage in ZD media or 24 h in OP medium. To establish the specificity of Zn treatment, groups of ZD cells were treated with their respective control media for the last 24 h (ZDA) or normal cells were cultured with OP medium supplemented with Zn (OP-Zn). ZDA treatments partially normalized Zn levels in the Opti and restored or elevated apoA-I mRNA levels in the Opti or CHE system, respectively. Similarly, the OP-Zn treatment restored the cellular Zn and apoA-I mRNA levels. Furthermore, one passage of culture with Zn-supplemented media in both the Opti and CHE systems resulted in higher cellular Zn and apoA-I mRNA levels than those for controls. Most significantly, short-term high-Zn induction to normal cells markedly elevated the cellular Zn (3-fold) and apoA-I mRNA (5-fold) levels. Data derived from this study strongly suggest that the expression of apoA-I is regulated by cellular Zn status.

The hypocholesterolemic effect of Zn deficiency, mainly the reduction of high-density lipoprotein (HDL) cholesterol, in animals (3, 9, 11) and humans (8, 14) has been documented. When the HDL fraction was examined by compositional and chromatographic analyses, Zn deficiency significantly reduced the total amount of plasma HDL particles, with no influence on the percent composition of total protein, triglycerides, phospholipids, and cholesterol (9). The reduction in the HDL cholesterol was mainly due to a marked decrease in apoA-I (apoE-)-free HDL, the major subclass of the HDL fraction (10). No alteration in very-low-density lipoprotein and low-density lipoprotein cholesterol levels was produced by Zn depletion.

In view of the association of low plasma HDL cholesterol and apoA-I levels with increased risk of atherosclerosis, the changes in lipoprotein metabolism induced by Zn deficiency may in part contribute to the development of atherosclerosis. Dietary Zn deficiency was found to be able to decrease the plasma apoA-I levels in both rats and hamsters (18). In addition, the hepatic apoA-I and metallothionein-II (MT-II) mRNA abundances were similarly altered by the Zn status. To further establish whether the observed alterations were truly due to Zn deficiency, groups of Zn-deficient (ZD) animals were given a Zn-adequate diet for 2 days. Such Zn replenishment either raised the plasma apoA-I level, as well as the hepatic MT-II and apoA-I mRNA abundances, to levels higher than control levels in rats or normalized them to the control levels in hamsters (18). These observations strongly suggested that Zn deficiency could specifically downregulate the hepatic expression of the apoA-I gene at a pretranslational step. The establishment of a suitable in vitro Zn deficiency model will facilitate the further investigation of the mechanism(s) responsible for such regulation.

In the present study, we have established three systems to manipulate the cellular Zn level in cultured Hep G2 cells, which could be used as in vitro models to study the influence of Zn status on human hepatic gene expression. In the first two systems, Hep G2 cells were cultured in two different low-Zn media to deplete cellular Zn over one passage, whereas in the third system, Zn depletion was achieved by exposing nearly confluent cells to a Zn-chelator for 24 h. Furthermore, the treatment specificity was established for each system by the replenishment of Zn. Moreover, long-term moderately high Zn treatment and short-term high-Zn treatment were also used as the Zn supplementation treatments. In all these systems, the cellular Zn levels and MT-II mRNA abundances were modulated by various treatments. Consistent with the results from in vivo studies, data derived from this study strongly suggested that Zn status may directly affect apoA-I gene expression.

MATERIALS AND METHODS
Cell culture and treatment. The human hepatoblastoma cell line Hep G2 was obtained from the American Type Culture Collection (Manassas, VA) and was used to mimic the human hepatic responses to different zinc statuses. All reagents were obtained from Life Technologies (Grand Island, NY). Cells were maintained in a regular medium composed of 90% DMEM-10% heat-inactivated fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Medium was replaced twice a week, and 7 days of culture were used as one passage. Nearly confluent cells at the end of passage 80 were subcultured at the ratio of 1:8 from a T75 flask to a 100-mm tissue culture dish and used as experimental cells.

Three different treatments were used to deplete cellular Zn from Hep G2 cells. In the first two experiments, Zn depletion
was achieved by culturing cells in low-Zn media for one passage. The Opti-MEM contains supplemented growth factors, and it is designed as a serum-free or serum-reduced medium for a DNA transfection assay (Life Technologies). A customized Zn-free Opti-MEM formulation was obtained from Life Technologies. In a preliminary study, Hep G2 cells were cultured for one passage in such medium, with 1% FBS and different levels of Zn addition (0 to 16 µM). The cellular Zn levels were altered in a dose-dependent manner (data not shown). In addition, no apparent morphological alterations could be observed within one passage of culture (data not shown). For the experiment, the ZD medium was prepared by adding 1% FBS to Zn-free Opti-MEM and was found to contain 0.4 µM Zn. The Zn-adequate (ZA4) medium was prepared by adding ZnSO₄ to the ZD medium to the level of 4 µM Zn (which was equal to the Zn level found in regular Hep G2 culture media with 10% FBS). The Zn-supplemented (ZA16) medium was prepared by adding Zn to ZD medium to the level of 16 µM Zn (which was equal to the Zn level found in the human plasma). Cells were cultured in normal medium for the first 12 h and then switched to ZD, ZA4, or ZA16 medium for the rest of the passage. A group of ZD cells were replenished for the last 24 h with the ZA4 medium and used as the ZDA cells. Four plates from each group were used for RNA isolation, and another four plates were used for the measurement of cellular Zn level.

In the second experiment, a divalent ion-chelating resin was used to remove Zn from FBS. Chelex 100 resin (Bio-Rad, Hercules, CA) was mixed with FBS at a 1:4 ratio (wt/vol), and the suspension was shaken for 2 h at 4°C, as described previously (5, 12). Chelex 100 resin was separated from FBS by centrifugation, and the extracted serum was further passed through a 0.4-µm filter for sterilizing serum and removing any remaining Chelex 100 resin. The resultant serum only contained the background level of Zn, as detected by atomic absorption, and the medium containing 90% DMEM and 10% such serum was considered Zn-free medium (basal medium). When cells were cultured directly in this basal medium, they could not reach levels of conjugacy that were the same as that for cells cultured in regular medium (data not shown), suggesting that cell growth was affected by the low-Zn status of the basal medium. The minimal supporting level of Zn was determined by a dosage curve, with the DNA content per plate as the index for the cell growth. At the low end (0.1–0.4 µM), the supplementation of Zn resulted in dosage-dependent increases in DNA content per plate (Fig. 1A; for clear illustration, only the data representing 0.2 and 0.4 µM are shown). However, the higher dosage of Zn supplementation resulted in essentially the same level of DNA content, which was comparable to that observed in cells cultured in the normal medium containing regular FBS (Fig. 1A). In contrast, the cellular Zn content exhibited continuous dosage-dependent increases over the entire testing range of Zn supplementation (Fig. 1B). These observations suggested that the basal medium containing 0.4 µM Zn could support the normal level of cell growth and maintain a depleted cellular Zn status. For the experiment, the ZD medium was the basal medium supplemented with 0.4 µM Zn. Similarly, the ZA4 medium and the ZA16 medium were prepared by adding 4 and 16 µM Zn to the basal medium, respectively, to mimic the Zn level observed in normal culture medium or in human plasma. Hep G2 cells were cultured either in ZD, ZA4, or ZA16 medium for one passage. As for the first experiment, a group of ZDA cells was also included. Four plates for each group were used for RNA isolation, and another four plates were used for the measurements of cellular Zn and DNA contents.

Fig. 1. Determination of optimal level of Zn in medium for depletion of cellular Zn from Hep G2 cells. Basal medium was prepared with 90% DMEM-10% Chelex 100-treated serum. Cells were cultured in basal medium supplemented with graded levels of Zn (only 0.2, 0.4, 1, and 4 µM levels are shown) for one passage. Another group of cells was cultured in normal medium and used as control. Cellular Zn and DNA contents were measured by atomic absorption spectrophotometry and diphenylamine procedure, respectively. Values are means ± SE from 4 experiments. Means with different letters are significantly different (P < 0.05 by 1-way ANOVA).

1,10-Phenanthroline (OP; Sigma, St. Louis, MO) is a Zn chelator widely used to remove Zn in cell-free systems, as well as to deplete cellular Zn from various types of cells (1, 7). In the third experiment of this study, OP was used to deplete cellular Zn from Hep G2 cells. Cells were cultured for 6 days in normal medium, and then 1% (vol/vol) of 100× OP stock, prepared in ethanol, was added to the culture medium. The minimal effective dose of OP was determined by dosage curves. As depicted in Fig. 2A, cells exposed to graded levels of OP for 24 h experienced dose-dependent reductions of cellular Zn contents. However, higher doses of OP (300 and 400 µM) may also affect the cellular metabolism, as evidenced by the significant decreases of DNA content per culture plate. Therefore, 200 µM was selected as the suitable dose and used for further evaluation of the optimal treatment time. In the time course assay, the Zn depletion effect of 200 µM OP was not exhibited until 18 h of treatment, and it was not further enhanced by extending the treatment to 36 h (data not shown). Thus 24 h appeared to be the optimal treatment duration. To examine whether any possible changes caused by the OP treatment are specifically due to the low-Zn status or other possible side effects of OP, increasing amounts of Zn were added back to OP-treated cells to counteract OP's Zn depletion action. As depicted in Fig. 2B, Zn replenishment resulted in dose-dependent increases in cellular Zn levels. Zn at 40 µM was found to be able to cause the reversion of the cellular Zn level to that of the controls. Although the cellular DNA content in cells treated with 40 µM Zn was slightly
to the regular medium supplemented with 200 µM ZnSO4 for 6 days. In the Zn induction (ZI) group, cells were then exposed formed. Cells were cultured in regular medium for nearly 7 expression, a short-term Zn induction experiment was per-
were used for RNA isolation.

were used for DNA and Zn analyses, and another four plates cultured in the normal medium for 6 days and then exposed to -ATG GAT CCC AAC TGC TCC TGC G-3

higher than that in unreplenished OP-treated cells, it was found not to be significantly different from that in the untreated control cells. The OP medium was prepared by adding 1% (vol/vol) 100× OP stock in ethanol to the regular culture medium. The control medium was prepared by adding the same amount of ethanol (OP carrier) to the regular

Values are means ± SE from 4 experiments. Means with different letters are significantly different (P < 0.05 by 1-way ANOVA).

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For controls, cells were cultured in normal medium, with same amount of ethanol used as OP carrier in OP medium and in OP medium supplemented with Zn (OP-Zn medium), for the last 24 h. Values are means ± SE from 4 experiments. Means with different letters are significantly different (P < 0.05 by 1-way ANOVA).

same amount of OP (A) or increasing amount of Zn-200 µM OP (B). For controls, cells were cultured in normal medium, with same amount of ethanol used as OP carrier in OP medium and in OP medium supplemented with Zn (OP-Zn medium), for the last 24 h. Values are means ± SE from 4 experiments. Means with different letters are significantly different (P < 0.05 by 1-way ANOVA).

were used for RNA isolation.

were used for DNA and Zn analyses, and another four plates were used for RNA isolation.

To further examine the effect of Zn status on apoA-I gene expression, a short-term Zn induction experiment was performed. Cells were cultured in regular medium for nearly 7 days. In the Zn induction (ZI) group, cells were then exposed to the regular medium supplemented with 200 µM ZnSO4 for 4 h, whereas the control cells were continuously cultured in the regular medium. The cells were then harvested, four plates for each group were used for DNA and Zn analyses, and another four plates were used for RNA isolation.

Determination of cellular contents of Zn and DNA. Cells were washed twice with PBS and harvested. After a centrifugation at 500 g for 5 min at 4°C, the cell pellet derived from one plate was resuspended in 1.5 ml PBS and sonicated. One milliliter of this sonicant was directly used to measure the cellular Zn content by means of a flame atomic absorption spectrophotometer (Hitachi), against the standard curve of 0.05–1.0 µg/ml of Zn (19). The remaining 0.5 ml of sonicant was used for the measurement of cellular DNA content (17). Total cellular contents of Zn per microgram of DNA are presented, because a linear relationship between the amount of cellular DNA and cell number, regardless of treatments, was previously established.

RNA isolation and analysis. In the first experiment (with Opti-MEM), total cellular RNA was isolated by using the TRIzol reagent (Life Technologies), according to the manufacturer’s instructions. The apoA-I mRNA abundance was then analyzed by Northern blot analysis, as described previously (19). 18S rRNA was used as the normalization reference. In all other experiments, RNA was isolated by using the RNAqueous kit (Ambion, Austin, TX), according to the manufacturer’s instructions. The abundance of apoA-I mRNA was measured by an RNAse protection assay (RPA) with the RPA-II kit (Ambion). To provide another index for the cellular Zn status and a positive control for Zn-regulated gene expression, MT-II mRNA abundance was also measured. The abundance of 18S rRNA was used as the internal reference for the normalization, and an 18S antisense template was purchased from Ambion. The RPA probe synthesized by T7 RNA polymerase was 128 nt in length. The 80-nt protected fragment of 18S rRNA appeared as double bands in RPA gels.

The human MT-II antisense template was prepared by RT-PCR, as previously described (15, 18). A pair of human MT primers, MT5 and MT3, corresponding to the 5’ and 3’ regions, respectively, of human MT-II cDNA (15), was synthesized. RT-PCR products were cloned into the pGEM-T PCR cloning vector (Promega). Plasmid DNA was isolated from a correct clone, which contained an MT-II cDNA fragment in the anti-

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Fig. 2. Determination of effective levels of 1,10-phenanthroline (OP) for depletion of cellular Zn (A) and of supplemented Zn for reversal of OP effect (B) in Hep G2 cells. Cells were cultured in regular medium for 6 days and then treated for another 24 h with media increasing amount of OP (A) or increasing amount of Zn-200 µM OP (B). For controls, cells were cultured in normal medium, with same amount of ethanol used as OP carrier in OP medium key and in OP medium supplemented with Zn (OP-Zn medium), for the last 24 h. Values are means ± SE from 4 experiments. Means with different letters are significantly different (P < 0.05 by 1-way ANOVA).

apoA-I antisense sequence.

A pair of primers, hAI-5 and hAI-3, was used to amplify a

A pair of human MT primers, MT5 and MT3, corresponding to the 5’ and 3’ regions, respectively, of human MT-II cDNA (15), was synthesized. The human MT-II antisense template was prepared by human apoA-I cDNA, a kind gift from Dr. Lawrence Chan (Baylor College of Medicine, Houston, TX). A pair of primers, hAI-5 and hAI-3, was used to amplify a 157-bp region (nt 364–520). The final cDNA template was 310 nt in length, and the RNA probe transcribed from the T7 promoter was 288 nt in length and contained 201 nt of human MT-II antisense sequence.

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RNA and probes were coprecipitated and resuspended in hybridization buffer and then hybridized overnight at 45°C. A diluted RNase cocktail was then added, and the mixture was further incubated at 37°C for 30 min. The RNase digestion was inactivated by the addition of inactivation buffer. The protected RNA probes were precipitated and separated by 6% acrylamide-8 M urea gel. To establish the specificity for the RPA signals, both a negative control and a positive control were included in each RPA gel. In the negative control, 5 µg of sample RNA were replaced with an equal amount of yeast RNA, and then the control mixture was processed through the same procedure as that used for the sample reactions. No protected signal could be detected in the negative control. The positive control was processed in the same manner as were the sample reaction mixtures but was not digested by RNase. Only full-length probes were observed in the positive control. The RPA gels were dried and autoradiographed. Band intensities of protected signals were quantified by a laser densitometer (Molecular Dynamics). The relative mRNA abundances in each sample were expressed as the arbitrary units of the apoA-I or MT-II band per arbitrary unit of the internal reference 18S in the same RPA reaction.

RESULTS

Zn depletion decreased the cellular apoA-I mRNA abundance. Significant reductions in cellular Zn levels were observed in cells from the Zn depletion treatment in all three systems. Compared with those of their respective controls, the cellular Zn level was reduced 55 or 48% by treatment with the ZD medium based on Opti-MEM or Chelex 100-extracted serum, respectively (Table 1). In addition, the OP treatment resulted in a 46% reduction in cellular Zn level (Table 1).

Consistent with the changes in cellular Zn levels, the cellular MT-II mRNA abundance was depressed by the Zn depletion treatments. As shown in Figs. 3 and 4, the MT-II mRNA levels were decreased to 38 and 43% of their respective controls by either one passage of culture in ZD Chelex 100 medium or 24 h of OP treatment. In Fig. 3, the reduction of MT-II mRNA abundance appeared not to be significant, primarily because of the highly elevated MT-II mRNA in ZDA and ZA16 groups. When the ZD- and ZA4-treated groups were analyzed separately, it was found that the reduction in MT-II mRNA was significant.

In addition to causing changes in the cellular Zn status and MT-II mRNA abundance, the Zn depletion treatments also significantly depressed apoA-I mRNA levels by 20 or 29% in the ZD cells cultured in the Opti-MEM system (Fig. 5) or in Chelex 100-based ZD medium (Fig. 3), respectively. Moreover, OP treatment also resulted in a 28% reduction in cellular apoA-I mRNA level (Fig. 4).

Table 1. Cellular Zn concentrations in various experimental groups

<table>
<thead>
<tr>
<th>Study</th>
<th>ZA4</th>
<th>ZD</th>
<th>ZDA4</th>
<th>ZA16</th>
<th>Control</th>
<th>OP</th>
<th>OP-Zn</th>
<th>Zn induced</th>
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<tbody>
<tr>
<td>Opti</td>
<td>1,396 ± 32†</td>
<td>628 ± 53§</td>
<td>1,127 ± 32†</td>
<td>2,286 ± 122*</td>
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<tr>
<td>Chelex 100</td>
<td>2,063 ± 129‡</td>
<td>1,080 ± 88§</td>
<td>1,678 ± 65‡</td>
<td>2,535 ± 142*</td>
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<tr>
<td>OP Induction</td>
<td>1,885 ± 106*</td>
<td>1,020 ± 30†</td>
<td>1,845 ± 51*</td>
<td>6,357 ± 193†</td>
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Values are means ± SE from 4 samples. Culture media and culture conditions are detailed in MATERIALS AND METHODS. For Opti and Chelex 100 studies, Zn-deficient (ZD) media contained 1% fetal bovine serum (FBS) in Opti-MEM or 0.4 µM Zn in DMEM containing 10% Chelex 100-extracted FBS, respectively. Zn-adequate (ZA4) and Zn-supplemented (ZA16) media were prepared by adding 4 and 16 µM Zn, respectively, to ZD media. Treatments lasted for 1 passage, and ZDA4 cells were ZD cells switched to ZA4 media for last 24 h. For 1,10-phenanthroline (OP) studies, control medium was regular culture medium containing 10% normal FBS. OP medium contained 200 µM OP, OP-Zn medium contained 200 µM OP-40 µM Zn, and Zn-induction medium contained 200 µM Zn. Treatments lasted for 24 h for OP study and 4 h for induction study. Values with different symbols are significantly different (P < 0.05 by 1-way ANOVA).
Alterations in cellular apoA-I mRNA levels appeared to be Zn specific. To examine the specificity of the Zn depletion effect on cellular apoA-I mRNA abundance, groups of ZD cells previously treated with ZD media were replenished for 1 day with their respective ZA4 control media. In the Opti-MEM system, such repletion elevated the cellular Zn level from 45% of control (ZD) to 81% of control in ZDA cells (Table 1). Although this level was still lower than that observed in ZA4-treated cells, the apoA-I mRNA level was already normalized to the control level (Fig. 5). Similar results were obtained from the Chelex 100 system. Compared with results for the unreplenished ZD-treated cells, ZDA treatment elevated the cellular Zn level from 52 to 81% of the control (Table 1). However, the same treatment significantly increased the cellular MT-II (448%) and apoA-I (174%) mRNA levels above those for the ZA4 controls (Fig. 3).

In the OP experiment, the addition of 40 µM Zn to the OP medium at the beginning of the OP treatment prevented the Zn depletion effect of OP, and the cellular Zn level was the same as that for the controls (Table 1). Consistent with the cellular Zn level, the cellular MT-II and apoA-I mRNA abundances were also not altered in OP-Zn cells (Fig. 4). These observations suggested that the reduction of apoA-I mRNA abundance in the OP cells did not result from possible side effects of OP.

Zn supplementation increased cellular apoA-I mRNA abundance. The influence of higher cellular Zn levels on apoA-I gene expression was also examined in the study. Because the Zn level in human plasma was found to be ~16 µM, this level was selected as the physiological level for Zn supplement. In the Opti-MEM culture system, cells cultured in ZA16 medium had a higher (164% of ZA4 control) cellular Zn content (Table 1). The ZA16 treatment also resulted in a small but significant increase (11%) in the cellular apoA-I mRNA abundance, compared with that for the ZA4 controls (Fig. 3). Accompanying the increased Zn level, the apoA-I mRNA abundance was coordinately increased to 173% of controls by the ZA16 treatment (Fig. 3).

To further test the concept that cellular Zn status may affect apoA-I gene expression, a short-term Zn induction experiment was performed. Cells were cultured in normal medium for the entire passage, and the ZI cells were treated with 200 µM Zn for 4 h before harvesting. Compared with results for untreated normal cells, ZI treatment did not affect the DNA content per plate (data not shown) but significantly increased the cellular Zn level to 312% of the control level (Table 1). Consistent with the changes in cellular Zn levels, MT-II mRNA and apoA-I mRNA abundances were also elevated 24- and 5-fold, respectively, in ZI cells (Fig. 6).
DISCUSSION

Previously, we have demonstrated that moderate Zn deficiency resulted in marked reductions in plasma total HDL apoA-I levels in rats and hamsters (18). In addition, the hepatic apoA-I mRNA abundances, together with the hepatic MT-II mRNA levels, were also downregulated by Zn deficiency in both rats and hamsters. Furthermore, a 2-day replenishment with a Zn-adequate diet of Zn-deficient animals normalized apoA-I levels in plasma and hepatic mRNA abundances to values that were the same as those for control hamsters and higher than those for control rats. These observations strongly suggested that Zn status regulated the hepatic apoA-I gene expression.

In this study, we have developed three methods to deplete cellular Zn from Hep G2 cells. The Opti-MEM system could deplete cellular Zn from Hep G2 cells, as well as from BHK cells (13), within one passage. However, when the treated Hep G2 cells were passed into the next passage, growth retardation was apparent after 3 days of culture in the second passage, regardless of the Zn concentration in the media (data not shown). These observations suggested that the Opti-MEM medium lacking Zn should only be used for certain applications and may not be suitable for a transfection assay in the second passage of future Zn depletion studies. This problem was largely avoided in the study using Chelex 100, in which 10% extracted serum was used. When cells were cultured in ZA4 medium, no morphological difference was observed over three continuous passages, compared with the cells cultured in normal medium (data not shown). In certain situations, a rapid short-term Zn depletion is desired, and the OP system can fulfill this need.

Besides the chelation of the Zn ion, Chelex 100 is capable of sequestering other divalent metals. However, the majority of these ions are toxic heavy metals, which should not exist in FBS or should exist at very low levels. To reduce the sequestering of other essential metals, only serum, not the final medium, was extracted. Moreover, all treatment media used in the Chelex 100 experiment were based on the same basal medium containing 10% extracted serum, and the only difference among all treatment media was the level of Zn added. Although the removal of other metals caused by Chelex 100 extraction may result in some possible influence on cellular metabolism, this influence should exist for all cells regardless of the treatment groups. Thus the observed treatment difference should be solely contributed by the Zn status.

Whenever cells are directly exposed to a chelator, possible side effects are major concerns. To address the question of whether the observed alterations in OP-treated cells were due to the Zn depletion or due to the side effects of OP, a predetermined amount of 40 µM Zn (Fig. 2) was added to the OP medium (200 µM OP) to counteract the Zn depletion effect. The cellular Zn and DNA contents, as well as the MT-II and apoA-I mRNA abundances, in OP-Zn-treated cells were all comparable to those for control cells, which were not exposed to OP. Thus the decreased cellular apoA-I and MT-II mRNA abundances associated with OP-treated cells were not caused by side effects of OP other than metal chelation. Similar to Chelex 100, OP has been reported to be capable of chelating other metals, although it is widely used as a Zn chelator. It is possible that the chelation of other ions may also contribute to the observed reduction of the apoA-I mRNA level in OP cells, and the addition of Zn to OP medium may block the chelation of both Zn and other ions by OP. To test this possibility, cellular Cu levels were also determined in OP-Zn-treated cells, because cellular Cu status is known to affect the apoA-I mRNA abundance. No difference in cellular Cu status among control, OP-, and OP-Zn-treated groups was detected (data not shown). In addition, we have also used as low as 20 µM Zn in another OP-Zn treatment during a preliminary study, and this dose was already capable of partially normalizing the reductions in cellular Zn, MT-II, and apoA-I
mRNA abundances caused by OP (data not shown). Therefore, the observed reduction of the apoA-I mRNA level in OP cells may be considered to have resulted mainly from Zn depletion.

Although the three methods used were largely different, Zn depletion by these methods resulted in marked reductions in cellular Zn, MT-II mRNA (not measured in the Opti-MEM experiment), and apoA-I mRNA levels. Moreover, Zn repletion was able to normalize all changes observed in Zn-depleted cells. These findings were consistent with the in vivo observations in Zn-deficient and Zn-replete rats and hamsters (18).

Zn status may regulate the apoA-I gene expression at several possible steps. In our previous studies, depletion of cellular Cu was found to increase the cellular apoA-I mRNA abundance in Hep G2 cells (19). This regulation was found to occur mainly at the transcription step, because the transcription rate measured by reporter gene transfection were markedly increased by the Cu depletion. In the present study, 200 µM Zn treatment was able to elevate the cellular apoA-I mRNA abundance to fivefold that of controls within 4 h. The half-time for apoA-I mRNA in normal Hep G2 cells was estimated to be ~44.5 h (19), and any possible changes in apoA-I mRNA stability should not have resulted in a large accumulation of mRNA within a short period of 4 h. Therefore, the elevated apoA-I mRNA abundance observed in the high-Zn-induced Hep G2 cells must have resulted from a large increase in transcription rate.

The metal-induced MT expression is mediated by the presence of multiple copies of metal responsive elements (MREs) in their promoters (6). These MRE sequences are small imperfect motifs, with a consensus MRE sequence of CTC^TGCRCNCGGCC (core sequence is underlined) in either orientation. Several nuclear factors are able to bind to MREs, and such bindings are enhanced by Zn (2). Besides the MT genes, a number of possible candidate genes responsive to metal regulation have been reviewed recently (4, 16). The initial search of the rat apoA-I promoter revealed a DNA sequence with a high degree of homology to the MRE consensus sequence (4). In addition, our laboratory has analyzed the human apoA-I promoter by using Transcription Element Search Software (TESS) against the database of known transcription factors. Several locations in the human apoA-I promoter were found to be capable of being recognized by known transcription factors, which mediated metal regulation. The DNA sequences around the positive transcription factor binding sites were further compared with the consensus sequence of MRE. One perfect MRE site and seven MRE-like sequences (with one mismatch) were identified (data not shown) as a cluster within the proximal apoA-I promoter (up to ~330 bp), similar to the MREs found in MT genes. The effect of cellular Zn status on interactions between these MRE-like regions and nuclear proteins needs to be analyzed by a gel mobility shift assay. In addition, the influence of cellular Zn status on the activity of the apoA-I promoter, with or without mutations at these MRE-like sites, also needs to be examined by a transfection assay.

In this study, both short-term Zn induction and long-term Zn supplementation altered the cellular apoA-I mRNA abundances, which were similar in trends but different in magnitudes. These observations suggested that a similar mechanism(s) may be involved in both cases. Thus short-term Zn induction can be used as an extension of a Zn repletion or supplementation system to provide a larger treatment effect, which may aid in the elucidation of the mechanism(s) responsible for the regulation of apoA-I gene expression by Zn status.

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