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ORIGINAL ARTICLE

# Significance of serum Zn-α2-glycoprotein for the regulation of blood pressure

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**Zn-α2-glycoprotein (ZAG) (molecular weight = 41 kDa) is one component in the α2 fraction of human plasma, and is reported to be associated with several diseases, such as cancers and metabolic syndromes. ZAG is also considered to be an important modulator of lipid metabolism. However, little is known about the correlation of serum ZAG levels with indicators of metabolic syndrome. Serum ZAG concentrations analyzed by enzyme-linked immunoassay were positively correlated with systolic and diastolic blood pressure in 326 subjects (236 males and 90 females) aged 17–79 years who had an annual health examination. By luciferase reporter and electrophoretic mobility shift assays, the core promoter region to regulate the *ZAG* gene expression was found to exist between −110 and −101. The transcription factor Sp1 interacted with this region, and Sp1 knockdown experiments showed that Sp1 critically regulated ZAG expression. Furthermore, ZAG increased the active form of RhoA, which was determined by pull-down assay. Increased serum ZAG concentrations induced, at least partly, by Sp1 may cause an increase in vascular tone through the activation of RhoA and contribute to elevated blood pressure.**
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**Keywords:** blood pressure; serum protein; small G protein; transcription factor

## INTRODUCTION

Zn-α2-glycoprotein (ZAG) is a glycoprotein present in the α2 fraction of human plasma and is precipitated with Zn ions with a molecular weight of 41 kDa.[1] In addition to human plasma, ZAG was also found in other human body fluids, such as saliva and cerebrospinal fluid.[2] We further demonstrated the existence of ZAG in a variety of normal human tissues by immunohistochemical analysis,[3] and first determined the nucleotide sequence of human ZAG cDNA.[4]

The biochemical and physiological properties of ZAG have been examined by many research groups. ZAG is secreted from adipocytes in both white and brown adipose tissues, and thus can be classified as an adipokine.[5,6] This glycoprotein plays a role in lipolysis in adipose tissues by stimulating adenylate cyclase through the β3-adrenoceptor.[7] ZAG seems to cause highly significant, time-dependent decreases in body weight without a reduction in food and water intake, suggesting the effectiveness of ZAG for the treatment of obesity.[8] Consistent with this, ZAG expression is downregulated in persons with obesity.[9,10]

The significance of ZAG expression has also been investigated in pathological states, such as cancer. ZAG was proposed to be associated with tumor differentiation of breast cancers and oral squamous cell carcinomas.[11,12] Recently, it has been shown that ZAG expression is enhanced in gastrointestinal cancer patients with cachexia,[13] a catabolic syndrome characterized by marked body weight loss and skeletal muscle atrophy.[14]

Considering these previous studies, ZAG appears to be an important modulator of lipid metabolism. However, the correlation of serum ZAG levels with indicators of metabolic syndrome, such as body mass index and blood pressure, in healthy human subjects has not been fully elucidated. In this study, we measured serum ZAG concentrations of participants undergoing health examinations, and investigated the correlation between serum ZAG concentrations and markers related to metabolic syndromes. We found that serum ZAG concentration was moderately and significantly correlated with blood pressure, and further examined the regulatory mechanism of ZAG expression and its possible involvement in the elevation of blood pressure.

## METHODS

### Participants and blood sample collection

All participants provided written informed consent to participate in this study, which was approved by the institutional ethical review board of Nagoya City University, Japan. Blood samples from 363 participants (265 males and 98 females) who took health examinations at the Hekinan City Medical Association, Aichi, Japan, were collected for measurement of biochemical

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markers and serum ZAG levels. The serum was separated from blood samples by centrifugation at $3000 \times g$ for 30 min, and stored at −80 °C until used.

### Reagents

Dulbecco's modified Eagle's medium was purchased from Sigma-Aldrich (St Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt was purchased from Roche (Basel, Switzerland). Protein G-Sepharose 4B and horseradish peroxidase-linked goat anti-mouse IgG were purchased from GE Healthcare (Piscataway, NJ, USA). Antibodies listed below were purchased from commercial sources: anti-Sp1 polyclonal antibody (pAb) and anti-RhoA monoclonal antibody (mAb) from Santa Cruz Biotechnology (Dallas, TX, USA), and anti-actin mAb from Sigma-Aldrich.

### Preparation of anti-ZAG polyclonal and monoclonal antibodies

pAb and mAb for human ZAG were prepared by the use of purified human ZAG protein as previously described.[15] Briefly, pAb for human ZAG was raised in New Zealand white rabbits by injecting purified ZAG (100 μg per rabbit) with Freund's complete adjuvant. The IgG fraction of antiserum was purified using a protein G-Sepharose column. mAbs for human ZAG were prepared by the fusion method with immunized lymphocytes and mouse myeloma cells. Purified ZAG (20–50 μg per mouse) was injected with complete Freund's adjuvant five times into 8-week-old BALB/c mice and spleen cells were collected under aseptic technique. Mouse myeloma cells and immunized mouse spleen cells were hybridized, and finally nine fusion cells were cloned. Supernatants from the nine clones (clones #1–#9) of hybridomas were, respectively, purified on the protein G-Sepharose column.

### Enzyme-linked immunoassay

Two-antibody sandwich immunoassays were performed. Wells of a F96 maxsorp immunoplate (Thermo Fisher Scientific, Pittsburgh, PA, USA) were coated with 100 μl of 20 μg $ml^{-1}$ affinity-purified ZAG pAb overnight at 4 °C. The wells were washed three times with phosphate-buffered saline (PBS), and blocked with 3% bovine serum albumin in PBS for 2 h at 4 °C. Serum from participants was diluted 200-fold with 3% bovine serum albumin/PBS, added to the wells in triplicates, and incubated for 2 h at room temperature. Wells were washed three times with PBS and incubated with 1 μg $ml^{-1}$ ZAG mAb (clone #1) for 2 h at room temperature. After washing three times with PBS, wells were incubated with horseradish peroxidase-linked goat anti-mouse IgG for 2 h at room temperature. Wells were washed with PBS four times and incubated with 100 μl of 0.3 mg $ml^{-1}$ 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) containing 0.03% $H_2O_2$ in 0.1 M sodium citrate buffer, pH 4.35, for 20 min at room temperature. The absorbance at 405 nm in each well was measured by a Multiskan JX microplate reader (Thermo Fisher Scientific).

### Cell culture

Human hepatoma-derived HepG2 cells, human prostatic carcinoma LNCaP cells, human embryonic kidney-derived HEK293 cells and rat vascular smooth muscle A7r5 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were maintained in a cell culture incubator at 37 °C with 5% $CO_2$ and humidified atmosphere. Cell passaging was performed at 80–90% confluence.

### Deletion analysis of the ZAG gene promoter region

The 5'-deleted promoters of human ZAG gene, cloned previously,[4] were inserted into a luciferase reporter plasmid (pGL4 vector; Promega, Madison, WI, USA), and analyses were conducted as previously described.[16] Briefly, the combination of each reporter plasmid (10 μg each) and the standard plasmid (6 μg) were transfected into HepG2 or LNCaP cells using FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. The cells were incubated for 48 h after transfection. The cells were lysed and the luciferase activity of the extracts was measured by luminometer (Lumi counter 1000; Microtec, Funahashi, Japan) with the PicaGene luminescence kit (Wako Pure Chemical Industries, Osaka, Japan). Values were standardized by the β-galactosidase activities measured in Multiskan MCC/340 M KII plate reader (Thermo Fisher Scientific) at 405 nm.

### Electrophoretic mobility shift assay

The probe and competitor for electrophoretic mobility shift assay were as follows: 5′-TATTAGGGGAGGAGCCCGTC-3′ (−110 to −91). The probe was labeled with [γ-$^{32}$P]ATP (GE Healthcare) and polynucleotide kinase (Toyobo, Osaka, Japan). The reaction mixture (20 μl) contained 6 μg protein of HepG2, LNCaP or HEK293 cell nuclear extract, 2 μg salmon testis DNA and 1 ng of probe ($10^4$ counts per minute) in Hepes-KOH buffer, pH 7.9. After incubation at 30 °C for 30 min, the reaction mixture was electrophoresed on a 4% polyacrylamide gel in 45 mM Tris-borate buffer, pH 8.3, containing 1 mM EDTA at 160 V for 30 min. The gel was dried and exposed to autoradiographic film overnight. For competition or supershift assay, 100 times molar excess of the competitor or 2 μg anti-SP1 pAb was separately added to the reactions 10 min before the addition of a $^{32}$P-labeled probe.

### Knockdown of Sp1

To knockdown the expression of Sp1 in HepG2 cells, the small interference RNA (siRNA) method was used as described previously.[17] Stealth RNAi duplexes against human Sp1 and a Stealth RNAi negative control duplex were purchased from Life Technologies (Carlsbad, CA, USA). The sequences of RNAi duplexes against human Sp1 were as follows: 5′-AAGAAUUUGUUGCU GCUGUGUCUGC-3′ (#1), 5′-UUGACAGGUAGCAAGGUG AUGUUCC-3′ (#2). siRNA transfection was performed using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. The cells were harvested 72–96 h after transfection.

### Western blotting

Cells were washed twice with ice-cold PBS and harvested using Lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 0.5% Nonidet P-40, 10 μg $ml^{-1}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 0.1 U $\mu l^{-1}$ benzonase). Then, 5× sodium dodecyl sulfate (SDS) sample buffer was added to cell lysates and the samples were boiled at 95 °C for 5 min. SDS samples were separated by SDS-polyacrylamide gel electrophoresis, followed by transferring proteins from the gel to polyvinylidene difluoride membranes, which were then incubated with the indicated Abs.

### Pull-down assay to assess RhoA activation

The pull-down assay was carried out as described previously.[18] To obtain ZAG for the stimulation of cells, HEK293 cells were transfected with a control vector (mock) or pFLAG-CMV5-ZAG using Lipofectamine 2000 (Life Technologies). Twenty-four hours after transfection, the cells were replated in serum-free Dulbecco's modified Eagle's medium/F12 medium. After a 72-h culture, the conditioned media (CM) were collected: ZAG concentrations in CM was approximately 10–50 μg $ml^{-1}$ as estimated by quantitative western blotting with ZAG pAb using purified ZAG protein as a standard. A7r5 cells were serum-starved for 24 h and stimulated with ZAG CM or mock CM as a control for 30 min. The cells were lysed in Buffer A (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 5% glycerol, 0.5% Nonidet P-40, 1 mM $Na_3VO_4$, 10 mM sodium fluoride, 10 μg $ml^{-1}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol) containing 30 μg of glutathione *S*-transferase-mDia-Rho-binding domain,[19] and then incubated at 2 °C for 30 min. The cell extract was obtained by centrifugation at $20\,000 \times g$ at 2 °C for 5 min, and then incubated with 50 μl of 50% slurry Glutathione-Sepharose beads (GE Healthcare) at 2 °C for 45 min. After the beads were washed with Buffer A, proteins bound to the beads were eluted with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis, followed by western blotting with an anti-RhoA mAb.

### Statistical analysis

Data are expressed as mean ± s.d. Correlations between serum ZAG concentrations and metabolic syndrome markers were analyzed by Microsoft Excel software. Difference in band densities was analyzed by the Student's paired *t*-test. $P < 0.05$ was considered to be statistically significant.

## RESULTS

### Clinical characteristics of participants

A total of 363 subjects (265 males and 98 females) who had an annual health examination agreed to participate in this study. However, 37 participants, who took medications, were excluded, because medications may affect the expression level of ZAG. Data from the remaining 326 participants (236 males and 90 females) aged 17–79 years were analyzed for serum ZAG concentration and clinical biochemical markers related to metabolic syndromes, and the results were shown in Table 1.

### Significant correlation between serum ZAG concentration and blood pressure

Analysis of a relationship between serum ZAG concentration and clinical biochemical markers was performed in 326 participants. Although previous studies indicated a significant correlation between ZAG and body weight, we did not observe a correlation from the data of the current study participants (Figure 1). ZAG is also considered a modulating factor for lipid metabolism, and we examined a correlation between ZAG and lipid biomarkers. Although ZAG had a significant positive correlation with triglyceride, it appears to depend on the existence of some exceptional triglyceride concentration data. On the other hand, ZAG did not correlate with total or low-density lipoprotein cholesterol levels. Similarly, there was no correlation between ZAG and blood sugar levels. However, ZAG correlated significantly and positively with blood pressure in the parameters examined in this study.

### Transcriptional regulation of ZAG expression

To identify how ZAG expression is regulated, deletion analysis of the ZAG gene promoter region inserted into a pGL4 luciferase reporter plasmid was used to measure luciferase activity in the HepG2 hepatic cell line. This cell line was used because the liver is one of the major organs in which ZAG is produced.[3,20] Luciferase activity was dramatically reduced when the promoter region of −214 was replaced with that of −101 (Figure 2a). Then, focusing on the promoter region between −214 and −101, we performed similar analyses and observed a remarkable reduction of luciferase activity at the promoter region of −101 compared with that of −110 (Figure 2b), suggesting that the promoter region between −110 and −101 is critical for transcriptional regulation of ZAG expression. Similar results were also observed in LNCaP cells (Figures 2a and b), which were previously shown to express ZAG.[21] Detailed observation could find for differences in luciferase activity at the promoter region of −101 between HepG2 and LNCaP cells; the promoter region of −101 retains subtle luciferase activity in LNCaP cells, but not in HepG2 cells. The exact reason for this difference is unclear at this time; however, one possible explanation is that some transcription factors that could slightly increase the activity are expressed in LNCaP cells, but not in HepG2 cells. Nevertheless, as the luciferase activity at the promoter region of −101 was quite low in LNCaP cells and the activity was sharply decreased at the promoter region between −110 and −101 in both cells, the results may provide the notion that for the regulation of the *ZAG* gene transcription, the importance of the promoter region between −110 and −101 is independent of cell types.

Next, we examined how the promoter region is involved in transcription regulation. When the $^{32}$P-labeled probe (−110/−91) containing this region was incubated with the nuclear extract from HepG2 cells, a gel-shift was observed by electrophoretic mobility shift assay (Figure 3a, lane 2), and this shift was clearly lost in the presence of unlabeled probe as a competitor (Figure 3a, lane 3). Similar results were observed when nuclear extracts from LNCaP cells and HEK293 cells were used (Figure 3a, lanes 4–7). As the focusing promoter region is rich in GC, we considered that the Sp1 transcription factor might interact with this region. When anti-Sp1 Ab was added, the gel-shift induced by the $^{32}$P-labeled probe (−110/−91) was super-shifted in the nuclear extracts (Figure 3b, lane 4), indicating that Sp1 might form a complex with the probe and nuclear extracts.

To understand the significance of Sp1 in the regulation of ZAG expression, Sp1 was knocked down in HepG2 cells and the level of ZAG expression was examined. We used two siRNAs against Sp1 (siRNA #1 and #2). siRNA #2 more effectively inhibited Sp1 expression than siRNA #1 (Figure 4a). Using siRNA #2, we confirmed that ZAG expression was downregulated in HepG2 cells (Figure 4b), demonstrating that Sp1 plays a role in the regulation of ZAG expression.

### Possible mechanism of ZAG in the increase in blood pressure

A series of evidence has demonstrated that activated RhoA and the consequent RhoA-mediated rearrangement of the actin cytoskeleton that induces contractility of vascular smooth muscle are important for blood pressure elevation, and are thus related to hypertension.[22–24] On the basis of this evidence, we hypothesized that ZAG might promote the activation of RhoA in vascular smooth muscle cells. When A7r5 rat vascular smooth muscle cells were treated with ZAG-containing CM, the amount of GTP-bound activated RhoA was increased compared with that in cells treated with control CM (Figure 5). The results may provide the possibility that ZAG is involved in the elevation of blood pressure through RhoA activation.

**Table 1 Clinical characteristics of participants**

| | |
|---|---|
| Age (years) | 41.5 ± 12.5 |
| Male/Female (*n*) | 236/90 |
| Body weight (kg) | 63.5 ± 12.2 |
| BMI (kg m$^{-2}$) | 22.9 ± 3.3 |
| Systolic blood pressure (mmHg) | 129 ± 14 |
| Diastolic blood pressure (mmHg) | 75 ± 11 |
| Blood sugar (mg dl$^{-1}$) | 96.9 ± 25.3 |
| Total cholesterol (mg dl$^{-1}$) | 206.0 ± 38.0 |
| LDL cholesterol (mg dl$^{-1}$) | 109.5 ± 28.7 |
| Triglycerides (mg dl$^{-1}$) | 149.1 ± 157.0 |
| ZAG (μg ml$^{-1}$) | 67.3 ± 21.1 |

Abbreviations: BMI, body mass index; LDL, low-density lipoprotein; ZAG, Zn-α2-glycoprotein.
Data are indicated by mean ± s.d.

## DISCUSSION

In this study, we demonstrated that serum ZAG concentrations were positively correlated with blood pressure in subjects undergoing a health check. However, another recent study showed that serum levels of ZAG were decreased in patients with hypertension.[25] Considering the results in this recent paper, we re-analyzed our data by dividing the subjects into two groups: a hypertensive group (systolic blood pressure ⩾140 mm Hg or diastolic blood pressure ⩾90 mm Hg) and a normotensive group (systolic blood pressure <140 mm Hg and diastolic blood pressure <90 mm Hg). The hypertensive and normotensive groups included 81 and 245 participants, respectively, and the serum ZAG concentrations in the hypertensive and normotensive groups were 72.1 ± 20.0 and 65.8 ± 21.3, respectively. The serum levels of ZAG were significantly higher in the hypertensive group than the normotensive group ($P$ = 0.020). The relationship of serum ZAG

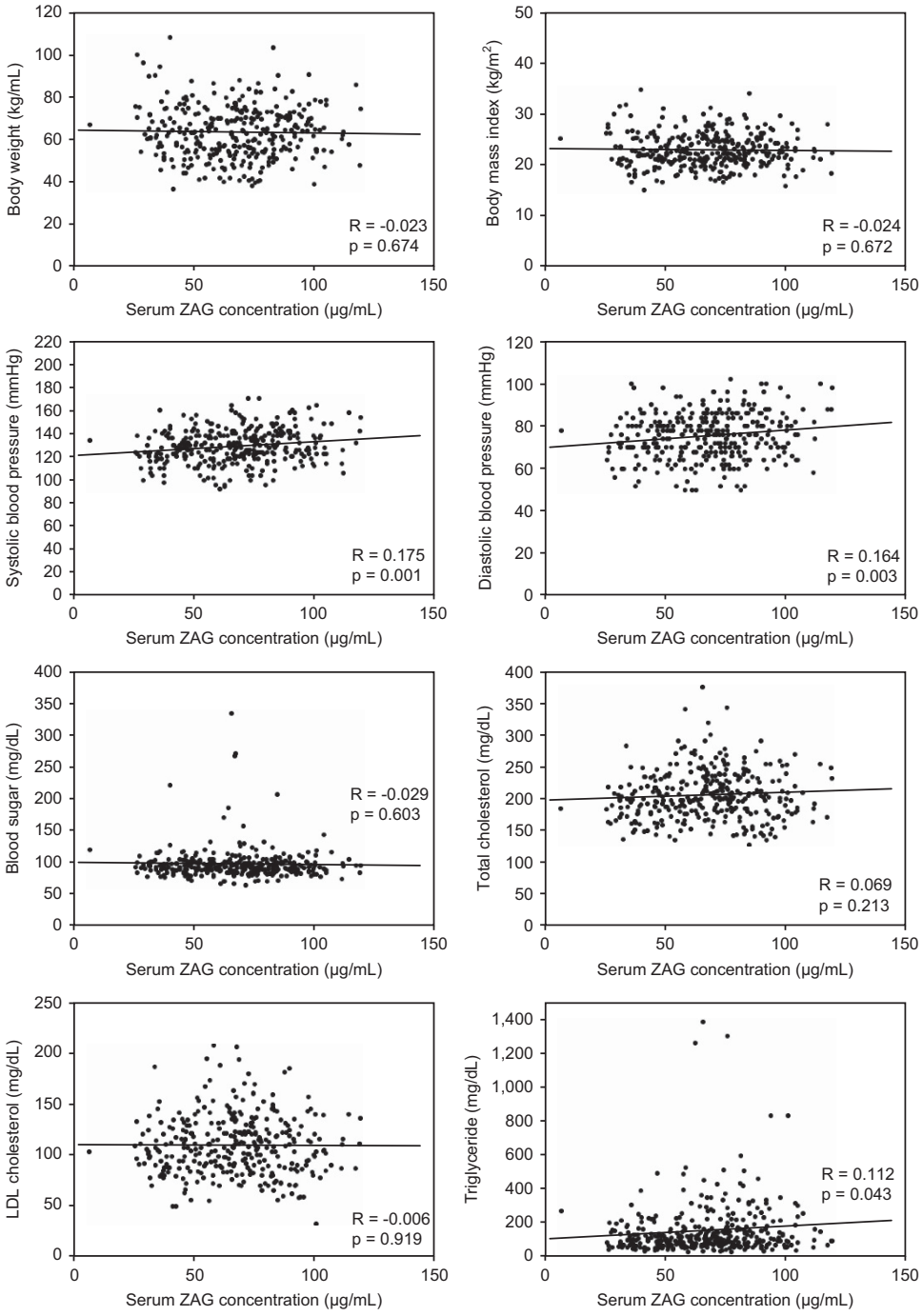


**Figure 1** Correlation between serum ZAG concentration and metabolic syndrome biomarkers in study participants.

concentration with hypertension is different between our study and the recent report.[25] An explanation for such a discrepancy is difficult, although different research conditions (in the previous report, newly diagnosed high blood pressure patients were examined) or differences in smoking and drinking habits that were not indicated in the previous report might explain the different outcomes between the two studies. Because the total number of subjects was high in our study (326 participants) compared with the previous study (74 patients), our conclusions might be more reliable.

There is some controversy regarding whether ZAG is related to obesity. In mouse models, administration of ZAG decreased body weight and fat without a change in body water or nonfat mass by increasing lipid mobilization and utilization.[8] Enhanced ZAG expression and secretion from subcutaneous adipose tissue was observed in cachectic cancer patients, and the secreted ZAG levels were significantly correlated with body weight loss.[13] Similarly, serum ZAG levels were elevated in pancreatic cancer patients with cachexia compared with those without cachexia.[26] ZAG-knockout mice had increased body weight in comparison with wild-type mice when fed identically.[27] Lipolysis induced by reagents, such as forskolin and isobutylmethylxanthine, was significantly attenuated in adipocytes from ZAG-knockout mice, which may contribute to weight gain.

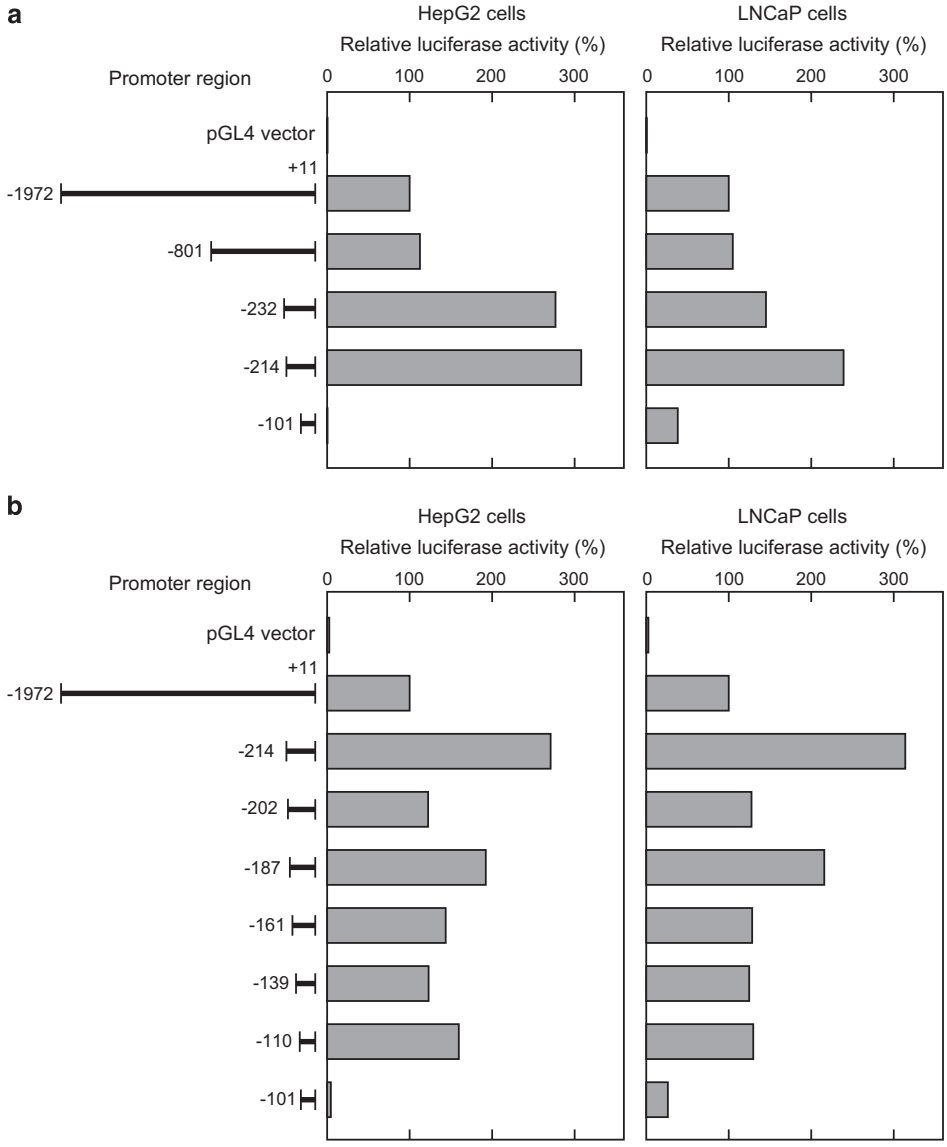


**Figure 2** Deletion analysis of the ZAG gene promoter region. (**a** and **b**) The left side shows the length of the ZAG promoter region, and the middle (HepG2 cells) and right (LNCaP cells) sides represent the relative luciferase activity. The values were compared with that of the promoter region (–1972/+11), which is arbitrarily expressed as 100%.

In contrast to these studies, another research group demonstrated that circulating ZAG levels were not significantly different in body mass index classification.[28] In the present study, we did not observe a correlation of serum ZAG with body weight or body mass index (Figure 1). The reason for inconsistency between these studies might depend on the different species used or pathophysiological states (cancer *vs.* relatively healthy persons).

Several previous articles showed that ZAG is associated insulin resistance or glucose tolerance,[29,30] whereas a study reported by Selva *et al.*[31] demonstrated that ZAG is unrelated to insulin resistance. On the basis of these literatures, the relationship of ZAG with diabetes mellitus or insulin resistance seems to be controversial, and thus, our results that serum ZAG concentration was not correlated with serum level of blood sugar may be understandable. As for the lipid metabolism, ZAG is recognized as a lipid-mobilizing factor and is implicated in lipolysis in pathological states, especially in obese animals and subjects. However, the data of ZAG in terms of its association with serum lipids in normal subjects are limited to date. Although it is a preliminary report, a recent study indicated that serum levels of ZAG were not correlated with serum low-density lipoprotein cholesterol and was weekly correlated with triglyceride ($P=0.035$) in healthy subjects.[32] The results are very similar to our findings of the present study. Another report also showed that in the normal glucose tolerance group, serum ZAG was not correlated with low-density lipoprotein cholesterol.[30] Considering these results, the effect of ZAG on lipid metabolism does not appear to be significant in the normal conditions.

We first identified the core promoter region that critically regulates the transcription of the *ZAG* gene, and also discovered that Sp1 is an essential transcription factor that interacts with the core promoter region to control transcription. In Sp1 knockdown experiments, ablation of Sp1 significantly reduced the expression of ZAG, but this reduction was not complete (Figure 4). Thus, other transcription factors and/or other transcriptionally regulatory mechanisms are likely

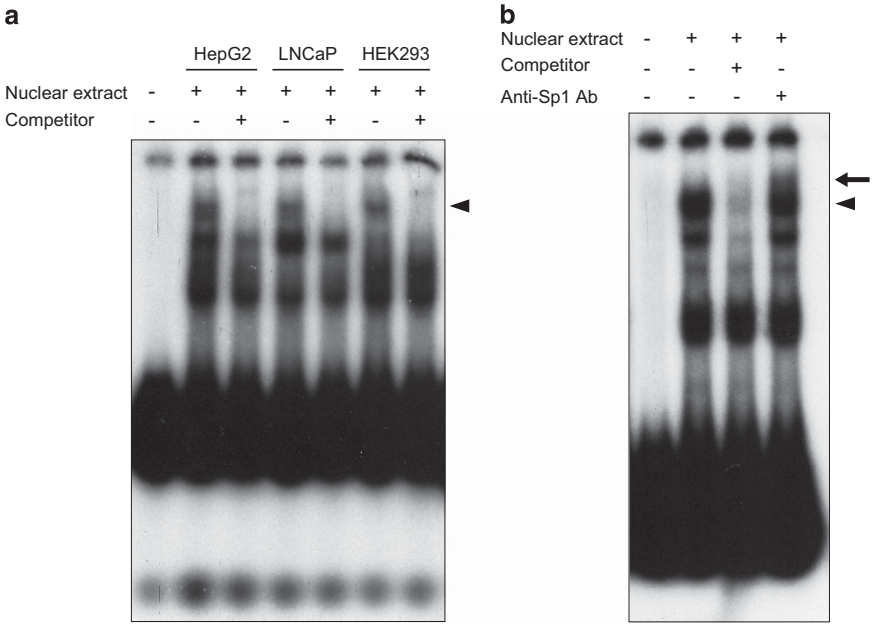


**Figure 3** Gel-shift patterns obtained by electrophoretic mobility shift assay. (**a**) Nuclear extracts obtained from HepG2, LNCaP and HEK293 cells were incubated with a $^{32}$P-labeled probe and ZAG promoter region (−110/−91), in the presence or absence of a competitor. The reaction mixture was electrophoresed by polyacrylamide gel, which was then subjected to autoradiography. (**b**) Similar experiments as described in (**a**) were performed using nuclear extracts obtained from LNCaP cells in the presence or absence of a competitor or anti-Sp1 Ab. Arrowheads (**a** and **b**) and arrow (**b**) indicate the shifted band and super-shifted band, respectively.

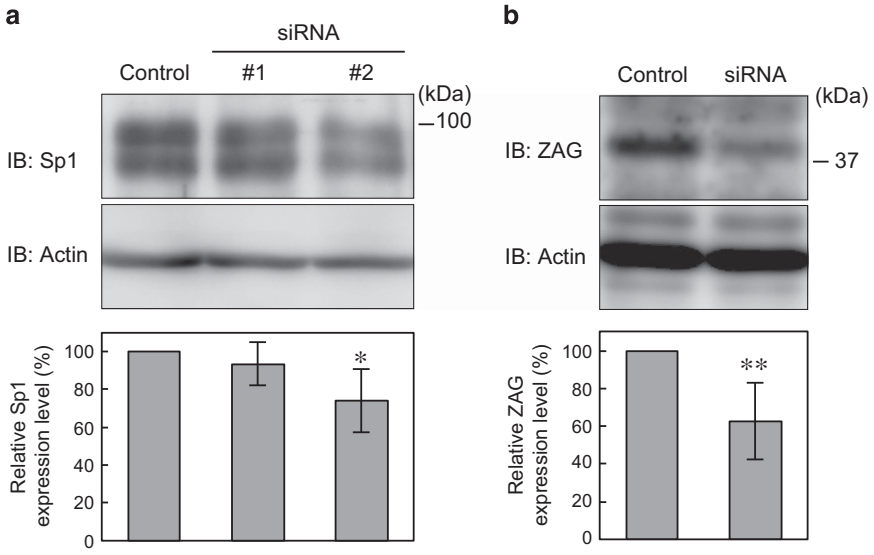


**Figure 4** Regulation of ZAG expression by Sp1. (**a**) Knockdown of Sp1. HepG2 cells were transfected with siRNAs against Sp1 (siRNA #1 and #2) or control siRNA. Knockdown of Sp1 was evaluated by western blotting with an anti-Sp1 Ab. (**b**) Inhibition of ZAG expression by Sp1 knockdown. HepG2 cell lysates transfected with control siRNA or siRNA #2 were used for western blotting with indicated Abs. Western blotting for actin (**a** and **b**) was conducted as an internal control. Bar graphs below the blotting images represent the relative expression of Sp1 (**a**) and ZAG (**b**) normalized to actin. The values were compared with control siRNA, arbitrarily designated as 100%. Data are means ± s.d. of five independent experiments. $^{*}P<0.05$; $^{**}P<0.01$ *vs.* cells transfected with control siRNA.

to be involved in *ZAG* gene transcription. These factors and mechanisms should be investigated in future studies. In addition, the signal pathways of how Sp1 is promoted to interact with the promoter region to upregulate *ZAG* gene transcription have not been determined yet in this study. The function of Sp1 is regulated by a large number of factors. Recently, the phosphorylation of Sp1 at Ser-59 was reported to be important for the induction of *CYP1A1* transcription in HepG2 cells.[33] It might be interesting to elucidate whether similar mechanisms contribute to the *ZAG* gene transcription regulation.

How ZAG expression is related to increased blood pressure was investigated using A7r5 vascular smooth muscle cells, and it was found

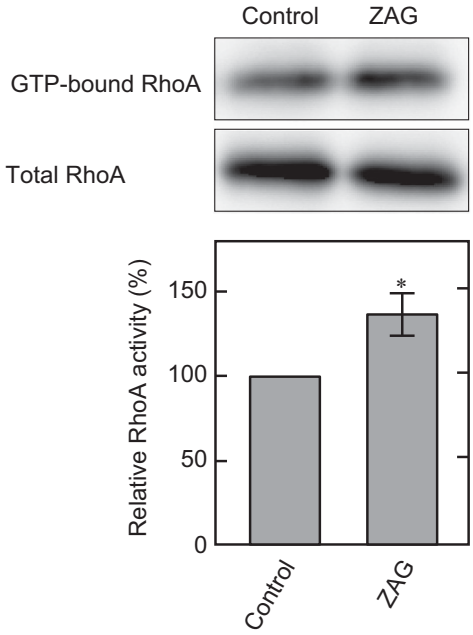


**Figure 5** Activation of RhoA in A7r5 vascular smooth muscle cells by ZAG. At 24 h after serum starvation, A7r5 cells were treated with ZAG or control CM for 30 min. Cell lysates were used for pull-down assay and subjected to western blotting using anti-RhoA mAb. Bar graphs below the blotting images represent relative RhoA activity calculated by the density of GTP-bound RhoA normalized to the total amount of RhoA. The values were compared with cells treated with control CM, arbitrarily designated as 100%. Data are means ± s.d. of three independent experiments. $*P<0.05$ *vs.* cells treated with control CM.

that ZAG facilitated RhoA activity in these cells (Figure 5). The activation of RhoA is important for the contractility of vascular smooth muscle cells, leading to an increase in vascular tone and elevation of blood pressure.[23,24] The molecular mechanism by which RhoA positively regulates the contractility of vascular smooth muscle cells is as follows: RhoA induces the activation of Rho-kinase, which inhibits the function of myosin light chain phosphatase and blocks the dephosphorylation of myosin light chain. Thus, the phosphorylation of myosin light chain relatively increases, resulting in the induction of contractility of vascular smooth muscle cells. Moreover, there are several lines of *in vivo* evidence that RhoA contributes to the elevation of blood pressure.[34–36] Given that ZAG can activate RhoA, an increase in ZAG concentration is likely to be the cause of blood pressure elevation, but not the result of it.

The precise signaling pathway of how ZAG activates RhoA is unanswered to date. Because ZAG exists in the extracellular fluid and there is no evidence that ZAG can pass through the plasma membrane, the machinery that transduces the ZAG-initiated signals to RhoA is prerequisite. At the first step, a specific cell surface molecule such as a receptor that recognizes ZAG might activate intracellular signaling molecules. Next, these molecules might cooperatively activate guanine nucleotide exchange factors to induce the activation of RhoA. We previously demonstrated that Vsm-RhoGEF, a guanine nucleotide exchange factors for RhoA, functions specifically in vascular smooth muscle cells and plays a role in the activation of RhoA.[18] Such guanine nucleotide exchange factors might also mediate the ZAG-initiated activation of RhoA. The identification of these molecular mechanisms should be investigated in future studies.

Because ZAG is a serum protein that appears to elevate blood pressure, it might be a good target for anti-hypertension therapy as clinical implications. The development of a ZAG inhibitor might contribute to the treatment of patients with hypertension having high serum ZAG concentrations, although the pathological situations and target organ damage levels widely vary among hypertensive patients.[37] This study may provide novel data for the development and progression of translational research in the field of hypertension.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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