High Glucose Induces Transactivation of the α2-HS Glycoprotein Gene Through the ERK1/2 Signaling Pathway

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Aim: Alpha2-Heremans Schmid glycoprotein (AHSG), also known as fetuin-A, is secreted from the liver and inhibits tyrosine kinase activity of the insulin receptor. Hyperglycemia in type 2 diabetes is not only a secondary manifestation of insulin resistance, but could also be responsible for directly inducing insulin resistance in target tissues. In this study, we examined the effect of high glucose (HG) on AHSG gene transcription in the human hepatoma cell line HepG2.

Methods: AHSG transcriptional activity and protein expression were evaluated using reporter gene assays and Western blot analysis, respectively.

Results: D-glucose, but not L-glucose or mannitol, dose-dependently enhanced AHSG promoter activity. HG (25 mM) also increased AHSG protein expression. No protein kinase C inhibitors (bisindolylmaleimide, Ro-31-8220), an inhibitor of hexosamine biosynthesis pathway (6-diazo-5-oxo-L-norleucine), or a superoxide radical scavenger (tempol) affected HG-induced transactivation. MAPK/ERK kinase inhibitors (PD98059, U0126), but not the JNK inhibitor (SP600125) or p38 inhibitor (SB203580), significantly inhibited promoter activation by HG.

Conclusion: Our data suggest that HG enhances AHSG transcription through activation of the ERK1/2 signaling pathway. Increased AHSG expression in the liver may be a cause of glucose toxicity in the diabetic state.


Key words; Alpha2-Heremans Schmid glycoprotein, AHSG, Fetuin-A, High glucose, Transcription
AHSG gene is located at chromosome 3q27, which has been identified as a susceptibility locus for type 2 diabetes and metabolic syndrome [15-17]. Recently, it has been shown that serum AHSG levels are significantly associated with insulin sensitivity in non-diabetic humans [18, 19]. These observations strongly supported the hypothesis that AHSG plays a physiological role in the regulation of insulin signaling and energy homeostasis.

If AHSG expression increases under hyperglycemic conditions, it could partly explain hyperglycemia-induced insulin resistance. Our study aimed to clarify the effects of high glucose (HG) concentrations on transcription of the AHSG gene, and its mechanisms in human hepatocyte cell lines.

**Materials and Methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin, and fetal calf serum were obtained from Invitrogen (Carlsbad, CA), and all tissue culture plasticware was from Nunc (Roskilde, Denmark). Human insulin was kindly provided by Novo Nordisk A/S (Bagsvaerd, Denmark). The PKC inhibitors bisindolylmaleimide (BIM) and Ro-31-8220 were from Calbiochem (La Jolla, CA). Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) inhibitors (MEK) inhibitors PD98059 and U0126, and the phosphoinositide 3-kinase (PI3-K) inhibitor LY294002 were from Promega (Madison, WI). c-Jun N-terminal kinase (JNK) inhibitor SP600125, p38 inhibitor SB203580 and glucosamine were from Sigma (St. Louis, MO). 6-diazo-5-oxo-L-norleucine (DON) was from MP Biomedicals (Irvine, CA), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), a stable spin trap for superoxide, was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Protein assay reagents were obtained from Bio-Rad (Hercules, CA), and nitrocellulose membrane from GE Healthcare (Buckinghamshire, UK). Polyclonal rabbit antibodies against human AHSG were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish-peroxidase conjugated secondary antibodies were from MP Biomedicals (Irvine, CA).

**Plasmid Constructs for Luciferase Assay**

The genomic sample was obtained from the whole blood of one of the authors (T.S.). The genomic DNA sequence spanning the AHSG gene was obtained from GenBank accession numbers D67013 and AC068631 and the transcriptional start site was determined according to the AHSG mRNA sequence (accession number NM 001622). For the luciferase reporter gene assays, a DNA fragment of the 5’-flanking region of the AHSG gene (−1091/+22) was amplified by the nest-PCR method using the following sense and anti-sense primers (5’-AGAGTTCTGC- CAGCAGCACC-3’ and 5’-GCAAGACAGAGGAG- CAGCAGC-3’) for the first DNA fragment, and (5’- GGGGTACCCTCTCCATGAGGGGCTTC-3’ and 5’-GAAGATCTCAGGCGTGAGTGTGTT- GGGC-3’) for nest-PCR, respectively. Italic capitals show the synthetic linkers, and underlined capitals show restriction sites for both Kpn I and Bgl II. The DNA fragments were loaded onto an agarose gel for electrophoresis and then purified using a gel extraction kit (Qiagen GmbH, Hilden, Germany). The purified DNA fragments were digested enzymatically (Toyobo, Tokyo, Japan) and inserted into a firefly luciferase expression vector, pGL4.12 [luc2CP] vector (Promega), which had already been digested enzymatically. The plasmid construct, pGL4-Fetu(A) (−1091/+22), was subcloned into JM109 bacterial cells, which was followed by isolation with a plasmid purification kit (Qiagen Plasmid Midi Kit; Qiagen GmbH). Sequences of the introduced DNA fragments were confirmed using a cycle sequencing method and an analyzer (BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and ABI PRISM™ 310 Genetic Analyzer), using the primers described for pGL4.12 and specific oligonucleotides within the inserted DNA sequence. The DNA insert sequence had adenine at position −799. For deletion analysis, various lengths of plasmids including −477/+22, −302/+22, −196/+22, and −89/+22 were generated using the same antisense and sense primers 5’-GGGGTACCCGGCCTTGGTCTCCCTCTGAGG-3’, 5’-GGGGTACCGTTCAACGCAGCAGGACCCGC-3’, and 5’-GGGGTACCGTTCAACGCAGGACCCGC-3’, respectively. DNA fragments were inserted into the firefly luciferase expression vector, pGL4.12 (Promega), and subcloned into JM109 bacterial cells. DNA fragments were confirmed by the cycle sequencing method.

**Cell Culture, Transfection, Treatment and Reporter Gene Assay**

Since transcription of the AHSG gene takes place predominantly in the liver, we used HepG2 cells, a human hepatoma cell line, in this study. The cells were cultured and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 20 µg/mL streptomycin.
Cultures were maintained at 37°C in a humidified 5% CO₂-containing atmosphere.

For transient transfection experiments, HepG2 cells, plated in 24-well plates, were transfected with the test plasmids and Tfx™-20 transfection reagents (Promega). The pRL-TK vector, which expressed Renilla luciferase (Promega), was co-transfected as an internal control. The final transfected DNA amount in each well was adjusted by the addition of empty vectors. Twenty-four hours after the start of transfection, the culture medium was replaced with serum-free and glucose-free medium, and the cells were then treated with the test substances for a defined time period. The final concentrations of reagents were 20 μM for DON, PD98059, U0126, SP600125 and SB203580, 1 mM for BIM, 1 mM for Ro-31-8220, 1 mM for tempol and 2 or 4 μM for glucosamine. When both D-glucose and the inhibitors were used, the cells were pretreated with the inhibitors and D-glucose was added 60 min later, and incubated for 24 h. When we examined the effects of insulin treatment on promoter activity, the cells were made quiescent by serum starvation for 24 h after transfection. At that time, insulin was added at 1–100 nM, and the cells were lysed after an additional incubation period. Both firefly and Renilla luciferase activities were measured using the Dual-Luciferase® Reporter Assay System (Promega).

Gel Electrophoresis and Western Blot Analysis
The cells were lysed in 300 μL buffer I (20 mM Tris acetate, pH 7.0, 0.27 M sucrose, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 10 mM benzamidin and 5 μg/μL leupeptin) per 3.5 cm dish. Supernatants were obtained by centrifugation at 15,000 rpm for 10 minutes at 4°C. The protein concentration was normalized, and 10 μg protein per sample was separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane, which had been blocked in 1× PBS buffer containing 0.2% Tween 20 (PBS-Tween buffer) and 3% milk powder, and subsequently in 1× PBS-Tween buffer containing 0.2% gelatin, prior to overnight incubation with polyclonal antibodies for human AHSG. Immunoreactive proteins were visualized using horse-radish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (ECL™) reagents.

Statistical Analyses
Each experiment was repeated at least three times to confirm the results. Results of the reporter gene assay are presented as the means ± S.E.M. For statistical analyses, data were compared by one-way ANOVA with Fisher’s protected least significant difference test. P values less than 0.05 were considered significant.

Results
Deletion Analysis of Upstream Region of the AHSG Gene
Fig. 1 shows luciferase activities of various lengths of the AHSG upstream sequence. Fragments 1 (F1: −1091/+22), 2 (F2: −477/+22) and 3 (F3: −302/+22) showed significant luciferase activity. F3 showed the highest activity; meanwhile, fragments 4 (F4: −196/+22) and 5 (F5: −89/+22) showed markedly reduced activity, suggesting an enhancer element between −302 bp and −196 bp. Relative luciferase activities of each fragment and pGL4-Basic, when mean luciferase activity of F1 was set as 100, were 100.0 ± 5.0 (F1), 167.5 ± 2.0 (F2), 358.5 ± 16.3 (F3), 5.3 ± 0.4 (F4), 2.4 ± 0.1 (F5) and 1.3 ± 0.1 (pGL4-Basic). Since it was unclear how D-glucose affects the AHSG promoter, we used F1 in later experiments, as it had the longest sequence of the fragments that showed significant promoter activity.
Effects of Glucose on AHSG Promoter Activity

To examine the effects of D-glucose on AHSG transcription, HepG2 cells were treated with 5–30 mM D-glucose. As shown in Fig. 2, D-glucose dose-dependently enhanced AHSG promoter activity. Although the dose-dependent effect and the fold induction of promoter activity by D-glucose varied depending on the cell condition, treatment with 25–30 mM D-glucose was found to produce more reproducible results than concentrations of 20 mM or less, and there was no significant difference in promoter activation between 25 and 30 mM D-glucose in repeated experiments. In a time-course study using 25 mM D-glucose, the highest promoter activity was observed when cells were incubated for 12 h, but there was no significant difference in promoter activity between incubation for 12 and 48 h (Fig. 3); therefore, in later experiments, we treated cells with 25 mM D-glucose for 24 h. Although fructose had a similar stimulatory effect on AHSG promoter activity, L-glucose and mannitol had no effect on AHSG promoter activity (Fig. 4).

Effects of HG on AHSG Protein Expression

To investigate whether HG-induced transactivation leads to the enhancement of AHSG protein expression, we carried out Western blot analysis and
found that HG (25 mM, 24 h) significantly enhanced AHSG protein expression (Fig. 5).

**Effects of Insulin on AHSG Promoter Activity**

We also investigated the effects of insulin on promoter activity. Following serum starvation for 24 h, cells were treated with 1–100 nM insulin for 1–24 h. Insulin showed no significant effect on AHSG promoter activity at any concentration or incubation period. No additive effect was detected when cells were treated with a combination of HG and insulin (data not shown).

**Mechanisms of HG-Induced AHSG Promoter Activation**

Hyperglycemia activates the DAG-PKC pathway and enhances oxidative stress, and they are closely connected. We therefore tested the effects of PKC inhibitors BIM and Ro-31-8220, and the radical scavenger tempol (Fig. 6) on AHSG promoter activation. BIM (5 μM) had no effects on basal or HG-induced promoter activity. Ro-31-8220 (1 mM) and tempol (1 mM) reduced both basal and HG-induced promoter activity, but did not reduce fold induction by HG; changes in fold induction by Ro-31-8220 and tempol were 2.4 to 2.9-fold and 2.7 to 3.9-fold, respectively. Since hyperglycemia also activates HBP, we next examined the effect of DON, an inhibitor of glutamine:fructose-6-phosphate amidotransferase, which is a rate-limiting enzyme of HBP. The result showed no effect of 20 μM DON on HG-induced promoter activity (Fig. 7, left). In accordance with this, treat-
The reporter gene assay was performed in HepG2 cells. The methods for transient transfection and the reporter gene assay are described in Materials and Methods. Cells were pretreated with 20 μM 6-diazo-5-oxo-L-norleucine (DON), D-glucose was added 60 min later, and then incubated for 24 h (left panel). Cells were treated with either 2 or 4 μM glucosamine (right panel). Basal promoter activity is set as 100, and relative activities are presented. Black bars show activity in cells incubated with 25 mM D-glucose, and white bars show activity in cells incubated with 5 mM D-glucose. Shaded bars show activity in cells incubated with 5 mM glucosamine and 2 or 4 μM glucosamine. Data represent the means ± S.E.M. (n=6). *p<0.0001.

![Fig. 7](image)

**Fig. 7.** Effects of inhibition of the hexosamine pathway on high glucose-induced promoter activation of the AHSG gene.

The reporter gene assay was performed in HepG2 cells. The methods for transient transfection and the reporter gene assay are described in Materials and Methods. Cells were pretreated with 20 μM 6-diazo-5-oxo-L-norleucine (DON), D-glucose was added 60 min later, and then incubated for 24 h (left panel). Cells were treated with either 2 or 4 μM glucosamine (right panel). Basal promoter activity is set as 100, and relative activities are presented. Black bars show activity in cells incubated with 25 mM D-glucose, and white bars show activity in cells incubated with 5 mM D-glucose. Shaded bars show activity in cells incubated with 5 mM D-glucose and 2 or 4 μM glucosamine. Data represent the means ± S.E.M. (n=6). *p<0.0001.

The involvement of the MAPK signaling pathway in HG-induced AHSG promoter activation was tested (Fig. 8). MEK inhibitors PD98059 (20 μM) and U0126 (20 μM) significantly inhibited the fold induction by HG; changes in fold induction by PD98059 and U0126 were 9.3 to 2.7-fold and 7.6 to 3.9-fold, respectively. Although the p38 inhibitor SB203580 (20 μM) reduced both basal and HG-induced promoter activity, it had no effect on fold induction (8.1-to 7.9-fold). The JNK inhibitor SP600125 (20 μM) elicited increased fold induction by HG (8.0-fold to 10.4-fold), while the PI3-K inhibitor LY294002 (20 μM) had no effect on fold induction by HG (data not shown).

To identify which region in the AHSG upstream sequence is crucial for the promoter activation by HG, we then compared the promoter activation in response to HG between F1 (−1091/+22), F2 (−477/+22), and F3 (−302/+22), all of which showed significant luciferase activity; however, no differences in both the fold induction of promoter activity by HG and the inhibitory effect of PD98059 were detected between fragments (Fig. 9).

**Discussion**

In this study, we demonstrated that D-glucose dose-dependently enhanced the transcriptional activity of the AHSG gene. Furthermore, we confirmed that, using Western blot analysis, this transactivation by HG leads to increased AHSG protein expression in HepG2 cells. Since L-glucose and mannitol had no effect on AHSG promoter activity, the effect of D-glucose on promoter activity was considered to be through its metabolic actions. Since hyperglycemia activates HBP and the DAG-PKC pathway, and enhances oxidative stress, we tested the effects of inhibitors of these pathways and a radical scavenger; however, we found that none of them affected HG-induced promoter activation.

We next tested the possibility that the MAPK signaling pathway, which is also associated with the glucose-stimulated intracellular signaling cascade, is involved in HG-induced promoter activation. MAPKs are a family of serine/threonine kinases that play an essential role in signal transduction by modulating gene transcription in the nucleus in response to changes in the cellular environment. MAPKs control key cellular functions, including proliferation, differentiation, migration and apoptosis. They include ERK1/2, JNK, and p38s, and each can be stimulated by a separate protein kinase cascade that includes the sequential activation of a specific MAPK kinase kinase and a MAPK kinase, which in turn phosphorylates and activates their downstream MAPKs. We found that MAPK/ERK kinase (MEK) inhibitors, but not p38 or JNK inhibitors, significantly inhibited HG-induced promoter activation. These results indicate that HG induces transactivation of the AHSG gene, at least in part, through the ERK1/2 signaling pathway; however, it remains unclear why insulin showed no effect on AHSG promoter activity, despite its well-known effect on the MAPK signaling pathway.

In deletion analysis, as the length of the promoter sequence shortened, luciferase activity increased, and F3 (−302/+22) showed the highest promoter activity, suggesting a repressive sequence upstream of this promoter region. On the other hand, F4 (−196/+22) and F5 (−89/+22) showed markedly decreased luciferase activity. This result indicates that an enhancer sequence is located between positions −302 and −196, which is consistent with a previous report by Banine et al. However, no difference in the fold induction of promoter activity by HG was detected between F1, F2 and F3, all of which had significant luciferase activity, suggesting that the sequence crucial for HG-induced promoter activation is located down-
stream of position -302. In this study, we could not identify the promoter sequence crucial for HG-induced promoter activation, since we could not form an accurate estimate of HG-induced activation because of extremely reduced promoter activity of F4 (-196/+22) and F5 (-89/+22). Computer analysis using TRANSFAC revealed that this region contains several putative binding sequences for transcription factors, including Elk-1, serum responsive factor, and c-Myc; therefore, further work is required to clarify which element is crucial for activation.

Despite the accumulating data regarding an association between AHSG and insulin resistance both in animals and humans, it remains unclear whether the serum concentration of AHSG is increased in diabetic patients. Ix et al. reported that an increased serum AHSG level was significantly associated with a high prevalence of diabetes mellitus. On the other hand, Mori et al. reported no difference in mean serum AHSG levels between non-diabetic and type 2 diabetic subjects. Transcriptional regulation in diabetes in vivo may be complex, since factors other than hyperglycemia (e.g. hyper/hypoinsulinemia and inflammation) could also influence AHSG gene transcription. In fact, it has been reported that interleukin-6, interleukin-1β and tumor necrosis factor-α downregulate AHSG synthesis. In addition, we did not examine the chronic effect of high glucose concentrations on AHSG transcription/expression, which may be different from the short-term effect.

Interestingly, fructose showed a similar enhancing effect on AHSG promoter activity (Fig. 4). We also found that this was a dose-dependent effect (data not shown). Fructose has detrimental effects on lipid metabolism and causes hepatic insulin resistance and increased serum fructose levels have been shown in diabetic patients; therefore, we should also inves-

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Fig. 8. Effects of inhibition of MAPK signaling pathways on glucose-induced promoter activation of the AHSG gene.

The reporter gene assay was performed in HepG2 cells. The methods for transient transfection and the reporter gene assay are described in Materials and Methods. Cells were pretreated with 20 μM PD98059, 20 μM U0126, 20 μM SP600125 or 20 μM SB203580. D-glucose was added 60 min later, and then incubated for 24 h. Basal promoter activity is set as 100, and relative activities are presented (upper). Promoter activity in cells incubated with 5 mM D-glucose both in the presence and absence of the inhibitors is set as 1, and fold induction of the promoter activity by 25 mM D-glucose treatment is also presented (lower). Black bars show activity in cells incubated with 25 mM D-glucose, and white bars show activity in cells incubated with 5 mM D-glucose. Data represent the means ± S.E.M. (n=6). *p<0.1, **p<0.001, ***p<0.0001.
In vivo studies, required to clarify the regulation of hepatic AHSG synthesis in diabetes.

**Conclusion**

We demonstrated for the first time that HG induces transactivation of the AHSG gene through the ERK1/2 signaling pathway in cultured hepatoma cells. Increased hepatic AHSG expression may partly explain glucose toxicity in diabetes.

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**Fig. 9.** Effects of high glucose and PD98059 on the promoter activity of various lengths of the AHSG promoter region.

The reporter gene assay was performed in HepG2 cells using pGL4-Basic vectors containing various lengths of the AHSG promoter region (−1091/+22, −477/+22, −302/+22). The methods for transient transfection and the reporter gene assay are described in Materials and Methods. Cells were pretreated with 20 μM PD98059, D-glucose was added 60 min later, and then incubated for 24 h. Promoter activity in cells incubated with 5 mM D-glucose both in the presence and absence of the inhibitors is set as 1, and fold induction of the promoter activity by 25 mM D-glucose treatment is presented. Black bars show activity in cells incubated with 25 mM D-glucose, and white bars show activity in cells incubated with 5 mM D-glucose. Data represent means ± S.E.M. (n = 4). *p < 0.05, **p < 0.001, ***p < 0.0001.
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