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Circ Res. Author manuscript; available in PMC 2009 September 9.

Published in final edited form as:

Circ Res. 2008 June 20; 102(12): 1558–1565. doi:10.1161/CIRCRESAHA.108.176990.

Aryl Hydrocarbon Receptor (AhR) is Activated by Glucose and Regulates the Thrombospondin-1 Gene Promoter in Endothelial Cells

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Abstract

Hyperglycemia is an independent risk-factor for development of diabetic vascular complications. The molecular mechanisms that are activated by glucose in vascular cells and could explain the development of vascular complications are still poorly understood. A putative binding site for the transcription factor Aryl Hydrocarbon Receptor (AhR) was identified in the glucose-responsive fragment of the promoter of thrombospondin-1 (TSP-1), a potent anti-angiogenic and pro-atherogenic protein involved in development of diabetic vascular complications. AhR was expressed in aortic endothelial cells (EC), activated and bound to the promoter in response to high glucose stimulation of EC. The constitutively active form of AhR induced activation of the TSP-1 gene promoter. In response to high glucose stimulation, AhR was found in complex with Egr-1 and AP-2, two other nuclear transcription factors activated by glucose in EC that have not been previously detected in complex with AhR. The activity of the DNA-binding complex was regulated by glucose through the activation of hexosamine pathway and intracellular glycosylation. This is the first report of activation of AhR (a receptor for xenobiotic compounds) by a physiological stimulus. This report links the activation of AhR to the pathological effects of hyperglycemia in the vasculature.

Keywords

aryl hydrocarbon receptor; glucose; thrombospondin-1; endothelial cells

Introduction

Hyperglycemia is an independent risk factor for vascular complications of diabetes[1–5]. Endothelial dysfunction is the earliest sign of developing diabetic vascular complications [for recent reviews, see[6–8]]. Hyperlgycemia affects the expression of numerous endothelial proteins[9–11], including thrombospondin-1 (TSP-1)[12], a potent anti-angiogenic and pro-atherogenic protein implicated in the development of a variety of vascular diabetic complications[12–15]. We report here that high glucose activates Aryl Hydrocarbon Receptor (AhR) in EC, which activates transcription of the thrombospondin-1 gene (*THBS1*).

AhR is a transcription factor known to be activated by aromatic hydrocarbons, e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) present in industrial waste, tobacco smoke and byproducts of herbicides[16–18]. Although the connection between AhR expression or activity and atherogenesis has not been explored directly, multiple epidemiological and animal studies

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Disclosures: None

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have established the association between known AhR activators and heart disease[18–23]. Recent reports demonstrated that AhR negatively affects angiogenesis in cancer and ischemia models[24–26], further implicating this transcription factor in regulation of endothelial function.

The abnormalities observed in AhR knockout mice include cardiac hypertrophy[27, 28], altered insulin regulation and responsiveness, altered glucose tolerance in pregnant females[29], and immune system impairment[30, 31]. Although AhR is clearly required for a variety of physiological processes[32–36], physiological activators of AhR are unknown, and only a few recent reports describe activation of AhR in response to pathological stimuli[24, 37, 38]. The mechanism of AhR transcriptional activity and the target genes have not been comprehensively studied[53,57–60]. There is no information on regulation of gene expression by AhR as a result of metabolic abnormalities.

Our results demonstrate that AhR is rapidly activated in EC in response to high glucose. Active AhR associates with the thrombosponsin-1 gene (*THBS1*) promoter and activates it. AhR forms a complex with several other transcription factors activated by glucose: AP-2, Egr-1, USF-2 and Pax-5. This complex is different from the complex formed by AhR and ARNT (HIF1β) in response to xenobiotics, and the activity of the complex is regulated by glycosylation.

This is the first report of AhR activation by high glucose that links AhR to the physiological regulation of gene expression by glucose and the pathological effects of hyperglycemia in the vasculature.

Materials and Methods

Cell stimulation with high glucose was described previously[12, 15].

Antibodies used

Anti-AhR from Novus Biologicals (Littleton, CO) and Abcam (Cambridge, MA), anti-Egr-1 from Cell Signaling Technology (Danvers, MA), anti-USF-1 and anti-USF-2 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), RL2 from Abcam (Cambridge, MA) and anti-AP2 from AbD Serotec (Raleigh, NC).

Promoter reporter constructs

The fragments −280/+66 pTHBS1 and −265/+66 pTHBS1 (ΔAhR) were generated by PCR.

Mutants: 1 - 5′AGCCCGCG**A**GGCGA3′, 2 −5′AGCCCG**GC**TGGCGA3′, 3 −5′ AGCCCG**GCA**GGCGA3′, wt - 5′ AGCCCGCGTGGCGCA 3′.

Analysis of the binding sites for transcription factors in the THBS1 promoter region responsive to glucose

The sequence of pTHBS1 was analyzed using MatInspector 7.4.3 (Genomatix, www.genomatix.de)[39].

Plasmids for the expression of AhR

The constitutively active form of AhR was prepared by constructing the AhR deletion mutant as described previously for murine AhR[40].

Analysis of activation of transcription factors in glucose-stimulated HAEC was performed using TranSignal Combo Protein/DNA array (Panomics).

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Immunofluorescence

Anti-AhR antibody (Novus Biologicals) and goat anti-mouse Alexa Fluor-labeled secondary antibody (Invitrogen) were used to stain sections of rat aorta[12].

Treatment of EC with glycosylation inhibitors and metabolites of hexosamine pathway was done as described earlier[15].

Statistical analysis

All the described experiments were performed more than 3 times and the data are presented as mean values ± S.E.M. P values were determined by T-test using Microsoft Excel. P values < 0.05 were considered statistically significant.

Results

The minimal fragment of human THBS1 gene responsive to high glucose in EC

We have reported recently that the expression of thrombospondin-1 (TSP-1) is increased in response to high glucose (10 – 30 mM) in all the major vascular cell types[12], and the increase in TSP-1 mRNA level is transcriptionally regulated[15]. The increase in mRNA levels could be detected as early as 1 hour after the start of stimulation in cultured EC and could be still detected at 72 hours in all vascular cell types[12]. We have analyzed the activity of TSP-1 promoter deletion constructs to identify the promoter elements responsible for this regulation in EC. The −280/+66 p*THBS1* fragment was activated in response to stimulation of HUVEC by 30 mM glucose (indicated by a 6-fold increase in activity of luciferase), and this activation was abolished by deletion of 15 base pairs in −265/+66 p*THBS1* (ΔAhR)(Fig. 1A), suggesting that a putative binding site for the transcription factor AhR predicted in this 15 bp region may control the response to high glucose. The response to glucose was inhibited in −380/+66 and longer promoter fragments, suggesting a presence of an inhibitory element in the promoter between −280 and −380, which is active in EC, but not in vascular SMC[15] or mesangial cells[41]. We analyzed the −280/+66 fragment of *THBS1* using MatInspector(Genomatix) to identify putative binding sites for transcription factors. This analysis identified several putative binding sites (Fig. 1B), including a binding site for AhR in the fragment responsible for glucose stimulation (−272, see Fig. 1B). The putative binding site for AhR overlapped with the predicted binding site for USF (−274), and this sequence was also recognized by the program as a Carbohydrate Response Element (CHRE).

Identification of transcription factors activated by high glucose in EC

We focused on transcription factors (TFs) that are rapidly activated in response to high glucose and are still active at 24 hours.

To identify the TFs rapidly activated in response to acute treatment with high glucose (1-hour stimulation with 30 mM glucose), we used four separate isolates of human aortic EC (HAEC). A targeted proteomic approach was used to identify the activated TFs. TranSignal Protein/DNA array (Panomics Inc., Fremont, CA) identifies only active TFs in nuclear extracts. Among the TFs activated in response to glucose treatment were six transcription factors whose putative binding sites have been predicted in the −280/+66 fragment of the *THBS1* promoter: AhR, AP-2, Egr-1, USF, NFkB, and Pax-5 (Table 1). We have confirmed the activation of Egr-1 and AP-2 using alternative methods of detection – Northern blotting and EMSA (see online supplement). The values in Table 1 were calculated based on increase in four independent experiments. We have chosen to analyze only the values for transcription factors activated greater than 2.5 fold in at least two out of four experiments. Activation of five out of six transcription factors was statistically significant, but the values for Pax-5 did not reach statistical significance. Figure 2A demonstrates representative array results for the five

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transcription factors significantly stimulated in response to high glucose in HAEC. USF, NFkB, and Egr-1 are known to be activated in response to high glucose or in diabetics[42, 43]. However, to our knowledge, this is the first report of activation of AhR and AP2 by glucose.

Confirmation of activation of AhR in EMSA

Nuclear extracts from HAEC and human umbilical vain EC (HUVEC) were used in an electromobility shift assay (EMSA) with a consensus AhR probe to confirm the activation of AhR by high glucose (Fig. 2B). Two DNA-binding protein complexes in both EC types were activated in response to 30 mM glucose (Complex 1 and Complex 2, Fig. 2B). When anti-AhR antibodies were used, they either prevented binding of both complexes to the probe or resulted in a supershift in EMSA. Unrelated antibody (AbX) was used as a control. When the probe corresponding to the sequence of the *THBS1* promoter containing the predicted binding site for AhR was used, activation and binding of AhR was also detected, and the binding was prevented by anti-AhR antibody (Fig. 2C).

AhR is expressed in EC

AhR is constitutively present in the cytosol and is translocated to the nucleus upon activation by its ligand[44–46]. We have detected AhR in both cultured HAEC and luminal EC of the rat aorta. (Fig. 3A, B).

AhR associates with TSP-1 promoter in glucose-stimulated HAEC

Chromatin immunoprecipitation from HAEC stimulated with 30 mM glucose for 1 hour was performed with anti-AhR antibody followed by the PCR amplification of the *THBS1* promoter region. It revealed that AhR associates with TSP-1 promoter only upon glucose treatment, while the general nuclear factor TFII is associated with the promoter in both control and glucose-treated EC (Fig. 4A).

Constitutively active AhR activates the THBS1 promoter

When the constitutively active form of AhR was co-transfected with the luciferase reporter/ −280/+66 p*THBS1* promoter construct in HEK293 cells, the activity of luciferase was increased more than 4 fold as compared to cells transfected with the control plasmid (Fig. 4B). The full-length AhR (inactive form) failed to induce any stimulation of the promoter (Fig. 4C).

In order to confirm that the promoter activity in response to active AhR is dependent on the binding site for this transcription factor (−272), we generated mutant −280/+66 p*THBS1* promoter constructs with one, two and three nucleotide substitutions in the core of the AhR binding site as described in Methods (Mut1, 2, and 3). The activity of these mutants remained at the basal level when co-transfected with active AhR (Fig. 4C). When HUVEC transiently transfected with these three mutants were stimulated with high glucose, none of the mutant −280/+66 p*THBS1* was activated in response to glucose (Fig. 4D), clearly confirming that the activation of −280/+66 p*THBS1* promoter by glucose depends on the AhR-binding sequence.

Complexes formed by AhR in high glucose-stimulated EC

Several transcription factors are known to form complexes with active AhR. The most common is HIF1β (ARNT) that associates with AhR in response to xenobiotic activators (reviewed in[47]); other proteins, including transcription factors, have been identified (reviewed in[45, 48]). We have used a TF/TF interaction array (Panomics) that detects only active transcription factors in complex with the transcription factor of interest and is based on the immunoprecipitation. The experiments revealed that AhR can be precipitated in complex with several transcription factors activated in response to glucose stimulation and having predicted binding sites in close proximity to the AhR binding site in the *THBS1* promoter. These are

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AP-2, Egr-1, USF, and Pax-5 (Fig. 5A), suggesting that these transcription factors may form a complex on the promoter of *THBS1* where the corresponding binding sites are in close proximity to each other in the region −280/+66. Neither of these proteins was precipitated with non-immune mouse IgG (data not shown).

To confirm these transcription factors form a complex in response to high glucose, we used corresponding antibodies in the binding reaction with consensus AhR probe (Fig. 5B, C) and TSP-1 probe (Fig. 5D). Formation of the specific complexes was prevented by anti-AhR antibody, while an unrelated antibody did not affect the binding (Fig. 5B). To characterize the complex of transcription factors, the following antibodies were used in combination with the AhR consensus probe in EMSA: anti-AhR (Fig. 2B, C; Fig. 5B, C, D), anti-Egr-1 (Fig. 5B, C, D), anti-AP2 (Fig. 5C, D), anti-USF-1 and anti-USF-2 (Fig. 5C, D). With the exception of USF-1, all these transcription factors were found in complex with AhR. Anti-AhR, anti-Egr-1, anti-AP2 and anti-USF-2 antibodies either prevented the binding of the complex to the labeled probe, or supershifted the complex band in EMSA. Anti-Egr-1 antibody prevented the formation of lower complex (Complex 2), but did not affect the binding of Complex 1 to the AhR probe, suggesting that Egr-1 is present in association with AhR in Complex 2, but Complex 1 is formed by AhR and other transcription factors (Fig. 5B). When TSP-1 probe was used, anti-Egr-1 antibody inhibited the formation of the complex. USF-1 antibody supershifted USF-1 protein in EMSA in an unrelated experiment (not shown). Antibody against HIF1β, the most common partner of AhR, did not affect either of the two complexes (data not shown) suggesting that HIF1β is not present in the complex.

Activation of DNA-binding complex is regulated by glycosylation

We have recently reported that in vascular smooth muscle cells intracellular glycosylation is responsible for activation of *THBS1* transcription[15]. We used RL2 antibody (anti-O-linked glucosamine N-acetyl, anti-O-GlcNAc), which recognizes glycosylated intracellular proteins, in EMSA to detect glycosylation of a protein(s) in complexes formed on the AhR consensus or TSP-1 probes (Fig. 5C, D). To confirm that glycosylation regulates the formation of the complex or its DNA-binding activity, we treated EC with: 1) 6-diazo-5-oxonorleucine (DON), which is an inhibitor of glutamine:fructose 6-phosphate amidotransferase (GFAT), an enzyme controlling the hexosamine pathway of glucose metabolism leading to formation of metabolites for glycosylation; 2) glucosamine, a glycosylation residue precursor, and a glucose metabolite entering the hexosamine pathway downstream of GFAT; 3) amino-N-phenylcarbamate (PUGNAc), known to increase O-linked protein glycosylation by effectively inhibiting β-N-acetyl-glucosaminidase (O-GlcNAcase), an enzyme responsible for cleavage of O-GlcNAc residues from intracellular proteins[15]. The inhibitor of GFAT prevented activation of the AhR complex (Fig. 6), and both glucosamine and PUGNAc resulted in activation of the complex without stimulation with high glucose, confirming that the activation depends on an intracellular glycosylation event. As expected, DON did not prevent complex formation in response to treatment of EC with glucosamine, which acts downstream of GFAT, confirming that the effect of DON was specific. PUGNAc did not induce the formation of the complex in the DON-treated cells, and this is consistent with our previous observations[15]: PUGNAc is an inhibitor of de-glycosylation, certain level of glycosylation is required in order to see its effect. However, in the DON-treated cells glycosylation is inhibited.

Discussion

Despite numerous studies on activation of AhR by xenobiotics[48, 49], its physiological activators, ligands and mechanism of activation remains unknown. Multiple indications of its role in normal physiology and pathology exist, including the AhR knockout mouse that develops metabolic abnormalities and cardiac hypertrophy[28–30]. Recent publications have

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reported a new role for AhR in response to UV exposure[37], modified low density lipoproteins[38] and hypoxia[24], further establishing its role in pathological changes.

Our results clearly demonstrate that glucose activates AhR in EC, and AhR controls the expression of TSP-1, a potent anti-angiogenic and pro-atherogenic protein. EC function determines physiological and pathological angiogenesis and initiation of atherosclerotic lesions in the large blood vessels. Therefore, activation of AhR by high glucose in diabetics or during postprandial elevation of glucose levels may initiate a series of pathological events leading to endothelial dysfunction and resulting in vascular disease. The epidemiological association between heart disease and industrial wastes and cigarette smoke that both contain activators of AhR also indicates a possible role for AhR in the development of atherosclerotic changes caused by environmental factors.

The role of AhR in the activation of the *THBS1* promoter was initially established by promoter analysis. The promoter region −280/+66 responsible for the increased transcription of *THBS1* was identified, and the AhR/USF binding site was predicted in this region, also recognized by MatInspector as a Carbohydrate Response Element (CHRE). The regulation of the *THBS1* promoter by high glucose in EC appears to be different from the regulation in mesangial cells[41] or vascular smooth muscle cells[27] where a longer promoter fragment is required for the response. The activation of AhR in EC was detected by a targeted proteomic approach and confirmed using EMSA. Furthermore, active AhR bound to the promoter of the endogenous *THBS1* gene in response to glucose. Overexpression of the constitutively active form of AhR but not the full-length AhR in together with the *THBS1* promoter/reporter construct further confirmed the activation of the promoter by AhR. The role of the AhR-binding sequence (−272) was confirmed using the mutant constructs with substitutions in the core sequence of the AhR-binding site: the mutant promoter fragments could not be activated in response to high glucose.

Gene transcription in response to extracellular and intracellular stimuli depends both on the promoter structure and on the signal- and cell type-specific patterns of activation of transcriptional activators, co-activators and suppressors. Transcription factors and co-activators form signal- and cell type-specific multi-protein complexes on the promoters. We have identified five proteins in complex with AhR in glucose-stimulated EC: AP-2, Egr-1, USF-2 and Pax-5. Interestingly but not surprisingly, the putative binding sites for all four transcription factors were predicted in the glucose-responsive fragment of the *THBS1* promoter in close proximity to the AhR binding site (MatInspector and[70]). Out of the multiple transcription factors with predicted binding sites in this region, only five were consistently activated (at least 2.5 fold) in response to high glucose in HAEC (AhR, AP-2, Egr-1, USF, NFkB), and four of them represented the proteins found in complex with AhR in both co-precipitation and supershift experiments with the corresponding antibodies. While the probe-based analyses did not allow to distinguish between USF-1 and USF-2 that both can bind to the same DNA sequence and form homo- and heterodimers, further analysis suggested that USF-2 is present in the complex, but not USF-1. Anti-USF-1 antibody that we used to supershift USF-1 complex in unrelated study with different cell type (data not shown) failed to change the binding of the AhR complex to both AhR concensus and AhR *THBS1* probe.

While the activation of Egr-1 and NFkB in EC and the activation of USF in other cell types and tissues in response to hyperglycemia or in diabetics was reported earlier[42, 50–534], this is the first report of activation of AhR and AP-2 by high glucose. Antibody against HIF1β did not affect AhR complexes in EMSA, and this transcription factor was not activated by high glucose in EC, suggesting that AhR-dependent transcriptional mechanisms activated in response to glucose differ from the well-described mechanisms activated by xenobiotics.

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Our data clearly demonstrate that activation of the transcriptional complex depends on glycosylation of at least one of the proteins: RL2 antibody recognizes proteins modified by O-linked N-acetylglucosamine, and this antibody supershifted the complex. Furthermore, inhibitors of the hexosamine pathway prevent formation of intermediates for intracellular glycosylation, and these inhibitors also prevent the activation of the DNA-binding protein complex, while the inhibitors of de-glycosylation and downstream intermediates of the pathway caused formation of the complex. Further confirmation that AhR can be directly glycosylated and the identification of glycosylation sites are clearly mandated by these observations.

The activation of SP1 in response to stimulation with high glucose was previously reported in both EC[54] and vascular smooth muscle cells[55]. SP1 undergoes post-translational modification by O-linked N-acetylglucosamine, which prevents its degradation in vascular smooth muscle cells[55]. Our array experiment did not detect any activation of SP1 in EC or in vascular smooth muscle cells (not shown), and our attempts to detect activated SP1 in nuclear extracts using a consensus probe were unsuccessful (data not shown). We believe that SP1 may be activated at the later time points, but not at the earlier time points analyzed in our studies. *THBS1* transcription is activated rapidly in response to high glucose[15], and this rapid activation suggests that only transcription factors activated before and at the onset of *THBS1* activation are involved in the regulation of the promoter in response to acute glucose stimulation.

The results of this work document a novel physiological and pathological role for AhR in the response of vascular EC to hyperglycemia. Despite the rapidly accumulating evidence that EC respond to glucose by activation of a variety of genes, the transcriptional mechanisms of this regulation have not been well explored, with the exception of a few reports identifying the specific transcriptional factors mediating a change in gene expression in response to glucose[42, 56–58].

This transcriptional mechanism provides a novel and unexpected link between hyperglycemia and the expression of TSP-1, a potent anti-angiogenic and pro-atherogenic protein involved in the development of multiple diabetic vascular complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Paul Bornstein (University of Washington) for cDNA of *THBS1* promoter, Dr. DiCorleto and Ms. Lori Mavrakis (Cleveland Clinic) for HUVEC, Dr. Priya Raman (Cleveland Clinic) for help with preparation of the biotinylated TSP-1 probe and advice on the design of TSP-1 promoter mutants, Ms. Yana Pleshivoy and Ms. Christy Harry (Cleveland Clinic) for technical help in preparation of the constructs and plasmids, and Mr. Tim Burke (Cleveland Clinic) for help with manuscript preparation.

Sources of funding

This work was supported by National Institutes of Health Grants K01 DK62128, P50 HL077107, R01 DK067532, American Heart Association grant 0565284B and funds from the Lerner Research Institute (Cleveland Clinic Foundation) (to O.I.S.).

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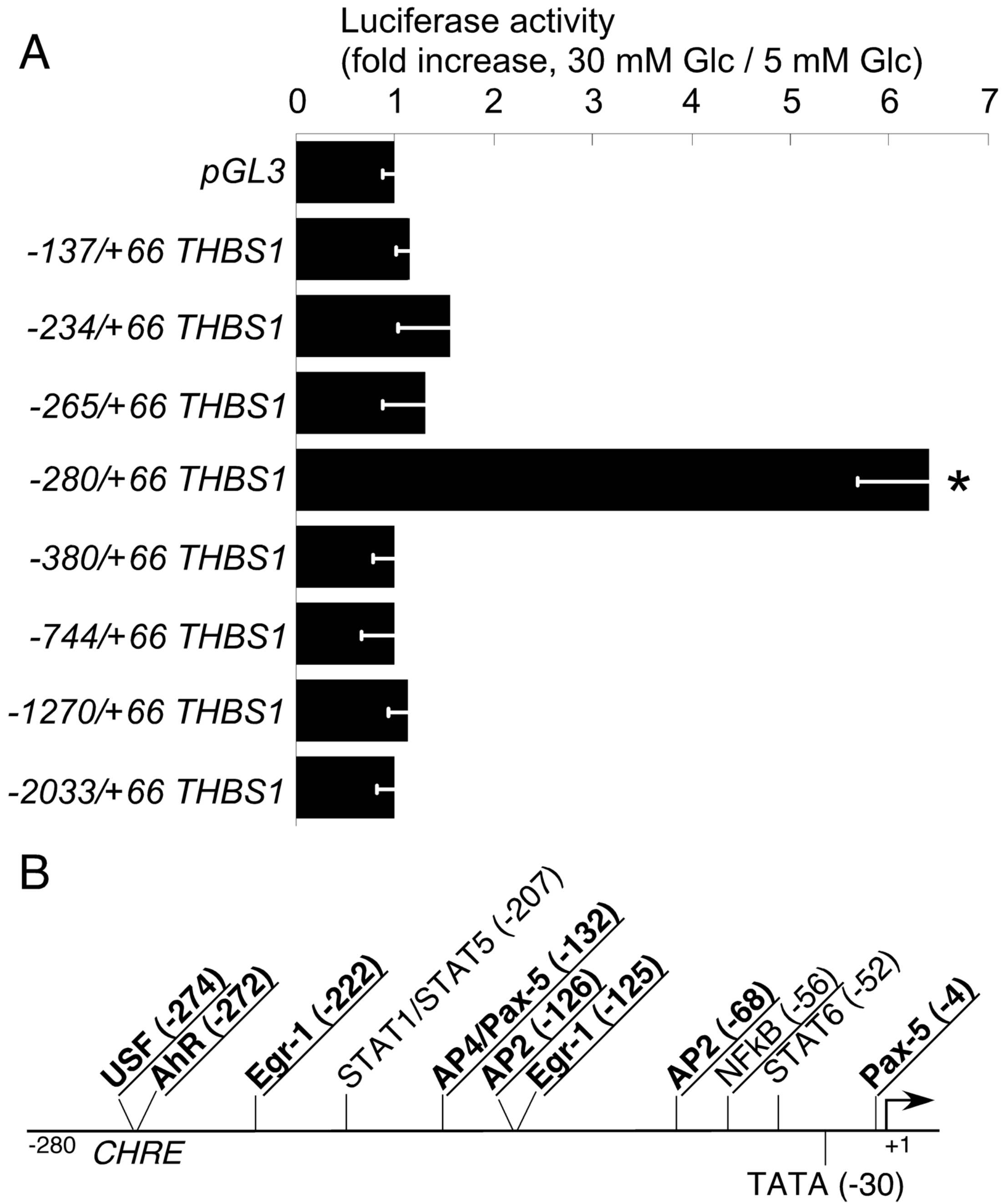

Figure 1. High-glucose-responsive promoter region

A HUVEC transfected with the promoter deletion constructs were stimulated with 30 mM glucose, luciferase activity was measured 24 hours later, n = 3, *p<0.05. **B:** Glucose-responsive region of the promoter was analyzed using MatInspector 7.4.3. to identify the putative binding sites for transcription factors. Transcription factors activated by high glucose are underlined, transcription factors co-precipitating with AhR in glucose-stimulated HAEC are in bold.

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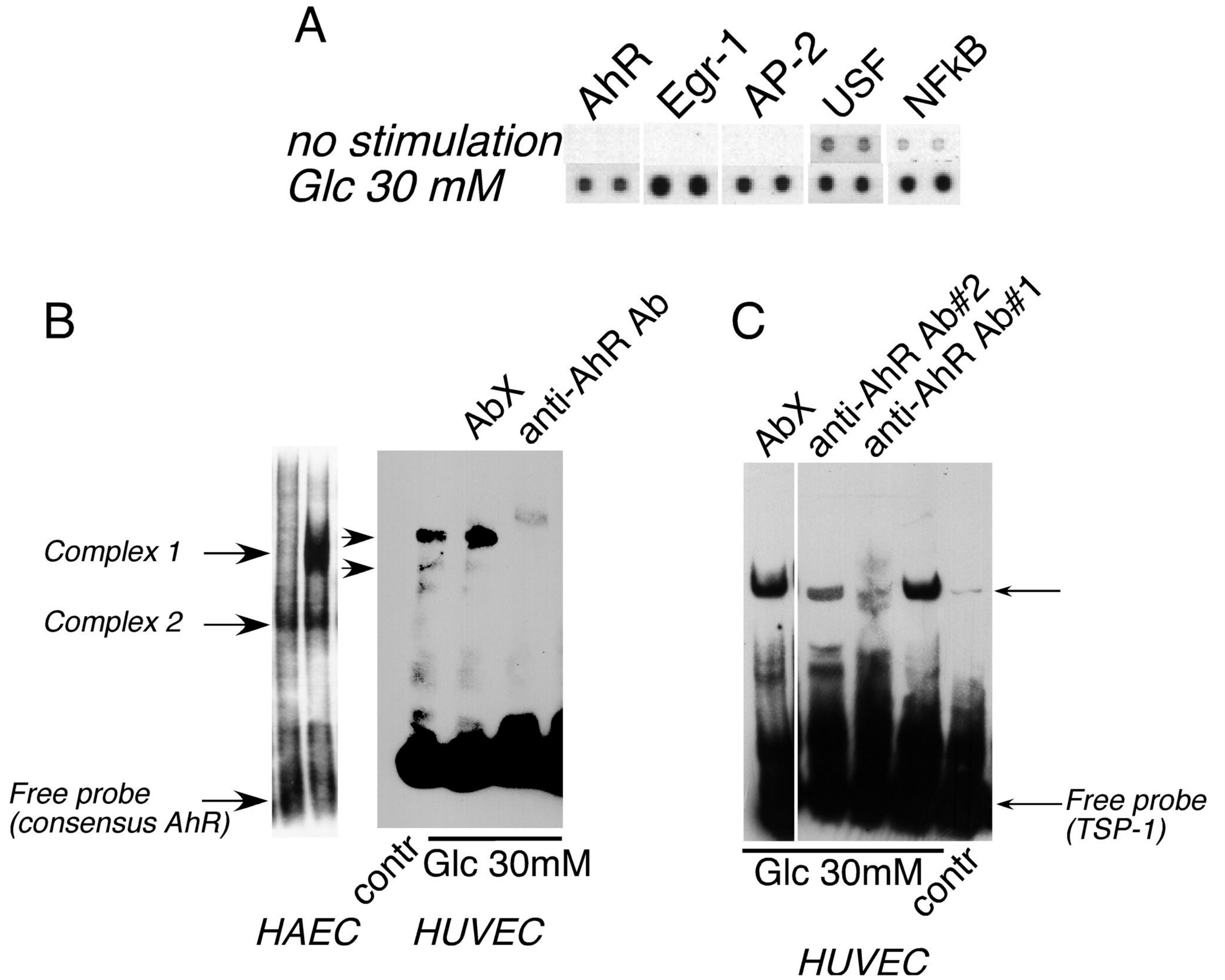

Figure 2. AhR is activated in endothelial cells in response to high glucose

A Nuclear extracts from HAEC stimulated with glucose for 1 hour were analyzed in a Protein/DNA array (Panomics) to detect activated transcription factors. Representative results for AhR, Egr-1, AP-2, USF-1 and NFkB (transcription factors with putative binding sites in the −280/+66 fragment of the *THBS1* promoter) are shown. **B:** Activation of AhR was confirmed in EMSA (5μg of nuclear extract) using the consensus AhR probe. Formation of complexes was prevented by anti-AhR antibody RPT1 (1 μg) but not an unrelated antibody (AbX). **C:** The predicted binding site for AhR in the promoter fragment responsive to glucose (−280/+66) was confirmed in EMSA using anti-AhR antibody.

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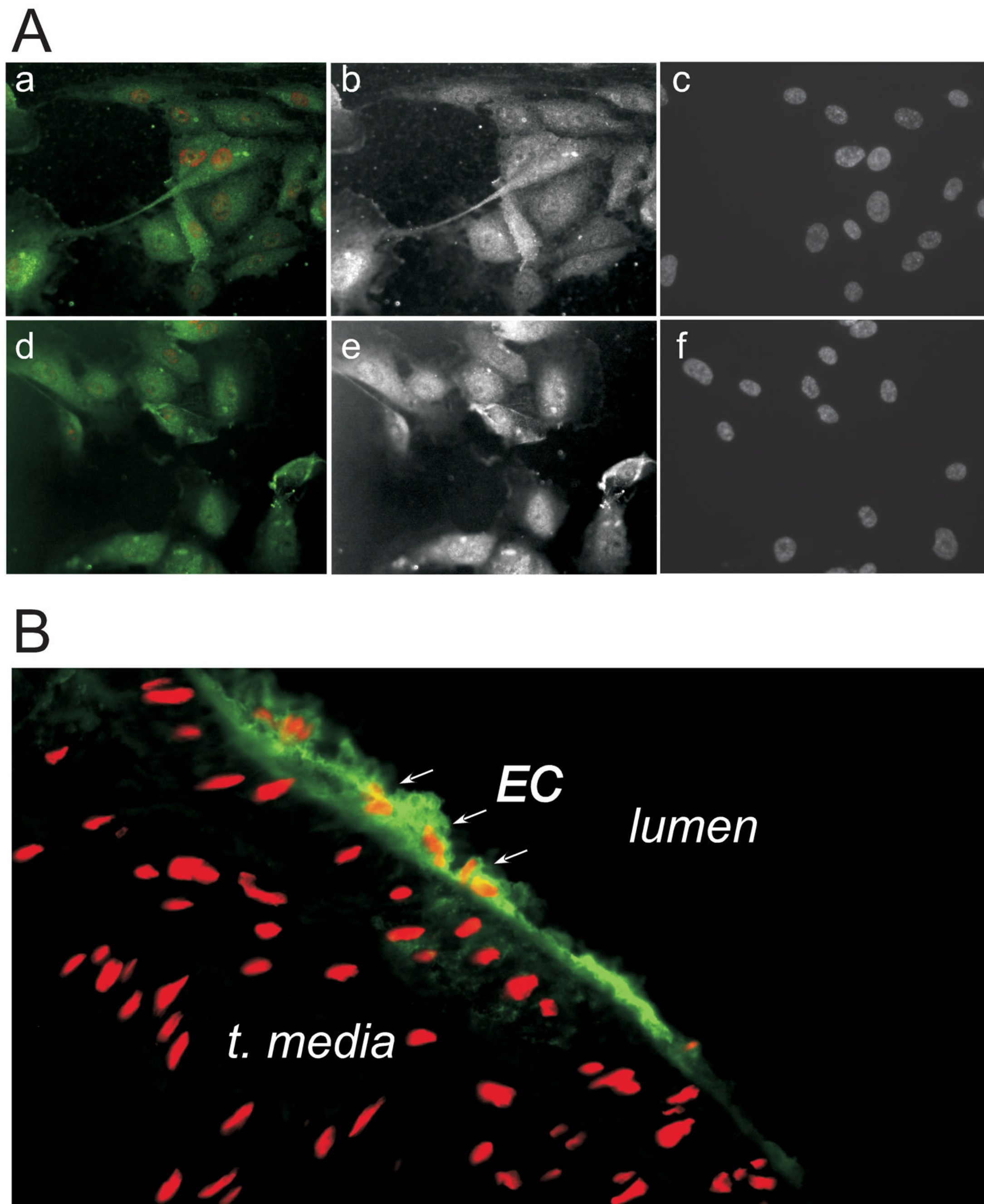

Figure 3. AhR is expressed in EC in vitro and in vivo

Cultured HUVEC (A) and rat aorta (B) were stained using anti-AhR antibody (green). Red – nuclei, propidium iodide staining. Aa-Ac: unstimulated HUVEC; Ad-Af: 3 hours of 30 mM glucose stimulation.

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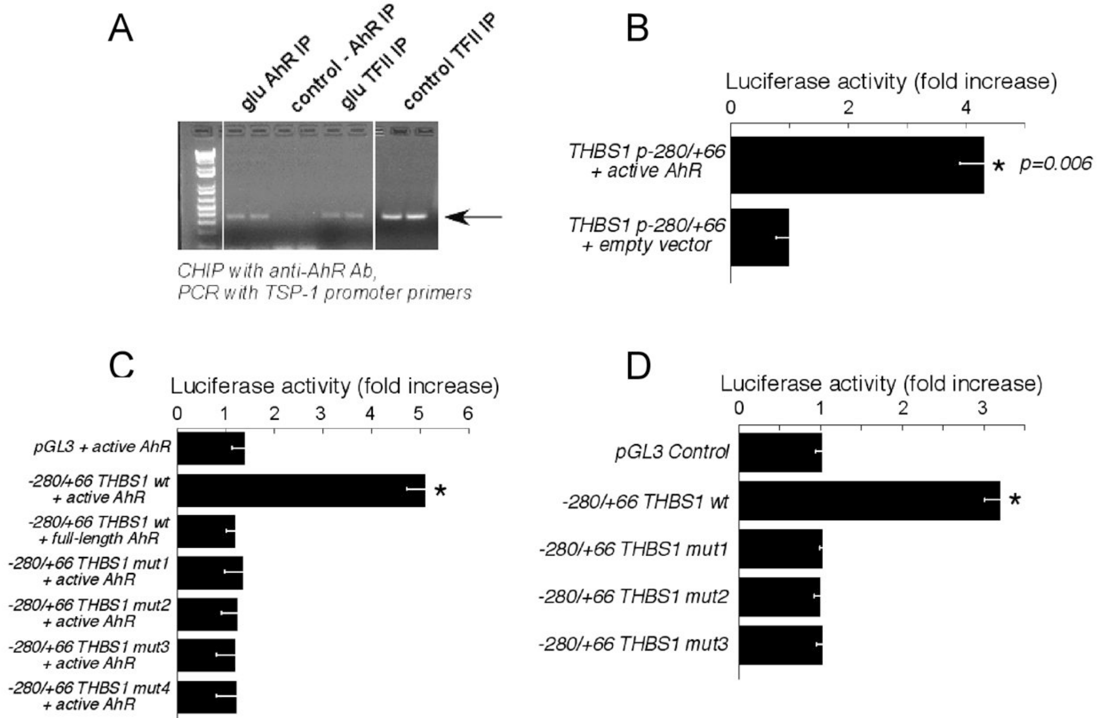

Figure 4. Active AhR binds and activates *THBS1* promoter

A HAEC (1 hour glucose) were used for chromatin immunoprecipitation with anti-AhR antibody. DNA from the precipitated fraction was used in PCR to amplify the *THBS1* promoter fragment; **B:** HEK293 were co-transfected with the −280/+66 *THBS1* fragment reporter plasmid and cDNA of constitutively active AhR, luciferase activity was measured 24 hours later. n = 3, *p < 0.05; **C:** HEK293 were co-transfected with indicated combinations of plasmids and the activity of luciferase was measured 24 hours later. Increase in the luciferase activity of the control, wt −280/+66 or mutant −280/+66 promoter constructs co-transfected with active or full-length AhR over the activity of the same promoter co-transfected with empty pcDNA3 is shown on the graph, n = 3, p < 0.05; **D:** HUVEC were transfected with the wt −280/+66 promoter fragment or mutant fragments and stimulated with 30 mM glucose for 24 hours; ratio of the luciferase activity in glucose-stimulated cells to non-stimulated cells; n = 3; *p < 0.05.

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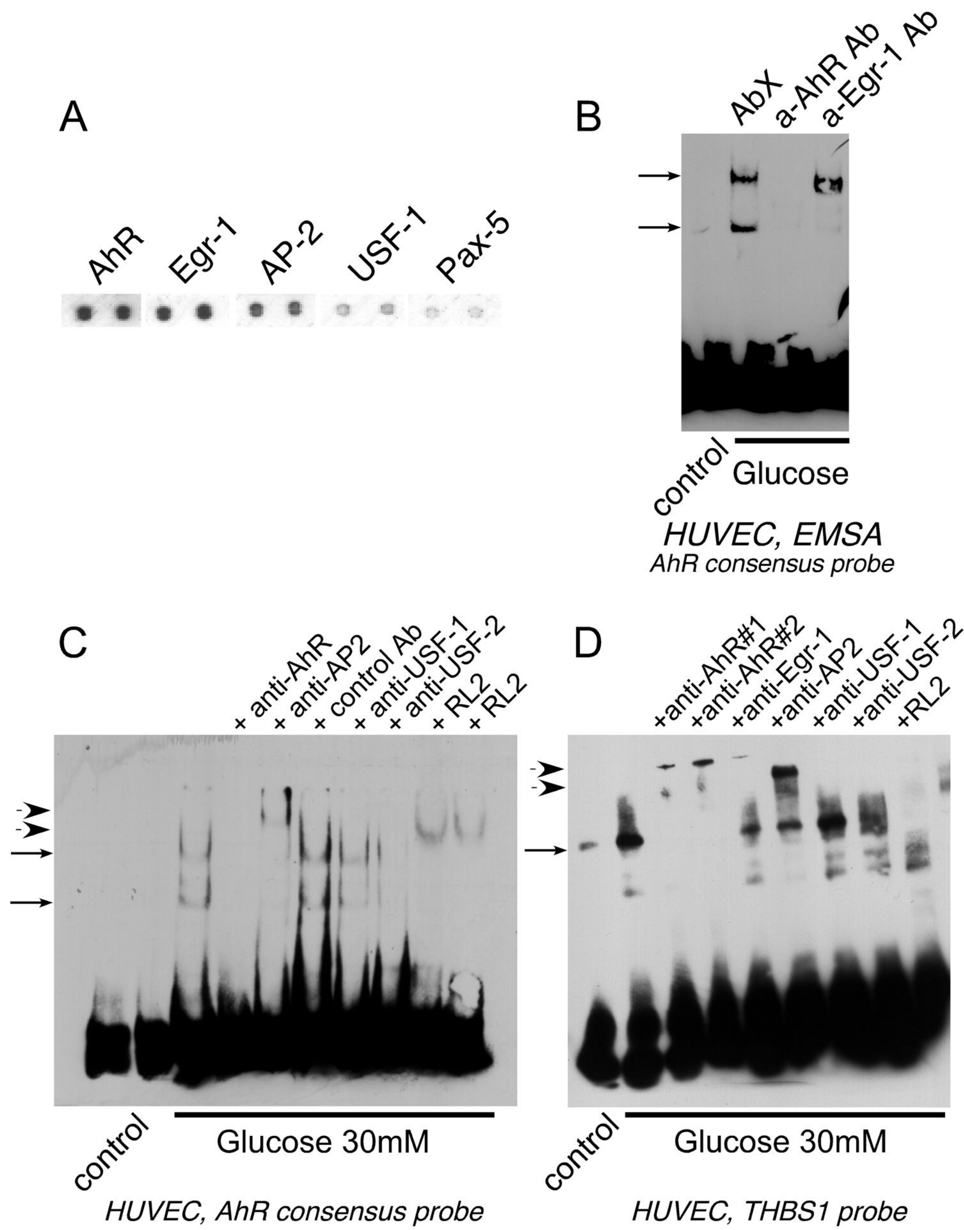

Figure 5. Transcription factors activated in EC by high glucose form a complex with AhR

A Nuclear extracts from glucose-stimulated HAEC were used for immunoprecipitation with anti-AhR antibody and associated active transcription factors were identified using TF/TF array (Panomics). **B:** Formation of a complex between AhR and Egr-1 in response to glucose was confirmed in EMSA using consensus AhR probe. One of the two complexes was prevented by the anti-Egr-1 antibody (1 μg). **C, D:** The complex of AP-2 and USF-2 with AhR was confirmed in EMSA using the antibodies against AhR (1 μg), AP-2 (1 μg), USF-1 (1 μg) and USF-2 (1 μg); C - consensus AhR probe, D - probe with the sequence of *THBS1* promoter fragment. RL2 antibody was used in both **C** and **D** to prove that at least one of the proteins is O-glycosylated.

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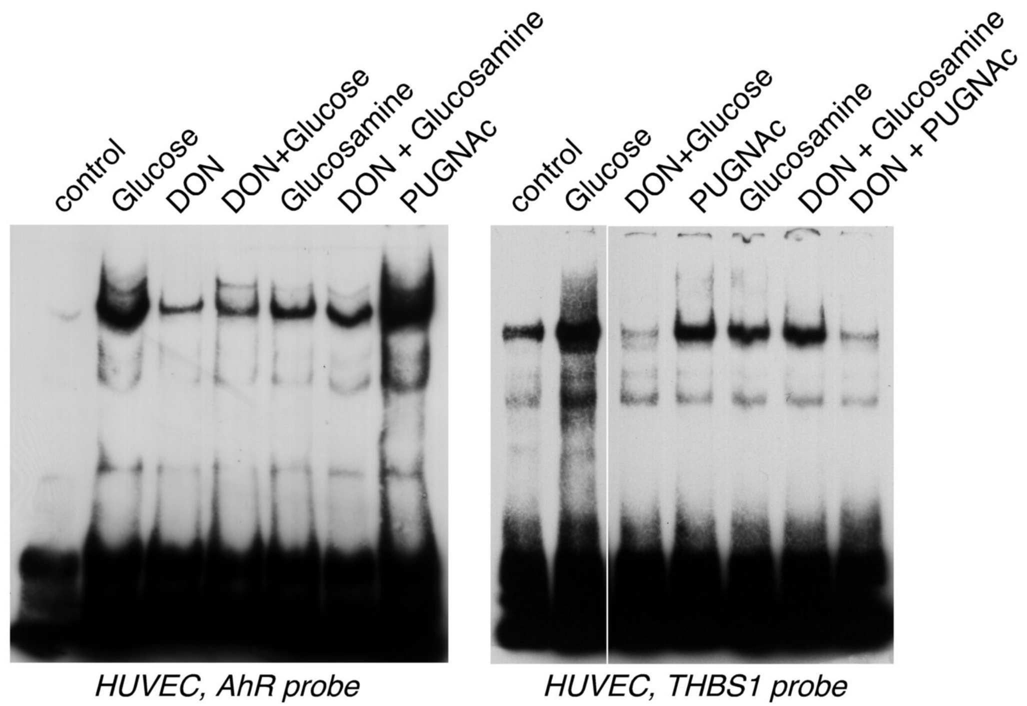

Figure 6. Glycosylation regulates the activity of the DNA-binding complex

5 μg of nuclear extracts from HUVEC treated for 24 h with 30 mM glucose, 1 mM glucosamine, 100 μM DON or 40 μM PUGNAc were used in EMSA with the consensus AhR probe (left panel) or TSP-1 probe (right panel).

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Table 1

Transcription factors with putative binding sites in −280/+66 p*THBS1* activated in response to high glucose in human aortic endothelial cells.

Transcription factor	Activation in response to 30 mM glucose (mean fold increase ± SE, n=4)
AhR	8.43 ± 2.3, p = 0.035*
AP-2	4.11 ± 0.57, p = 0.025*
Egr-1	3.65 ± 1.1, p = 0.035*
USF	2.8 ± 0.5, p = 0.05*
NFkB	3.75 ± 0.4, p = 0.01*
Pax-5	2.38 ± 1.28, p = 0.2