Aryl Hydrocarbon Receptor 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, a potent antiangiogenic and proatherogenic protein involved in development of diabetic vascular complications. AhR was expressed in aortic endothelial cells (ECs), activated, and bound to the promoter in response to high glucose stimulation of ECs. The constitutively active form of AhR induced activation of the thrombospondin-1 gene promoter. In response to high glucose stimulation, AhR was found in complex with Egr-1 and activator protein-2, which are 2 other nuclear transcription factors activated by glucose in ECs 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. **(*Circ Res*. 2008;102:1558-1565.)**

Key Words: aryl hydrocarbon receptor ■ glucose ■ thrombospondin-1 ■ endothelial cells

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Hyperglycemia is an independent risk factor for vascular complications of diabetes.[1–5] Endothelial dysfunction is the earliest sign of developing diabetic vascular complications (reviewed recently[6–8]). Hyperglycemia affects the expression of numerous endothelial proteins,[9–11] including thrombospondin (TSP)-1,[12] a potent antiangiogenic and proatherogenic 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 endothelial cells (ECs), which activates transcription of the TSP-1 gene (*THBS1*).

AhR is a transcription factor (TF) known to be activated by aromatic hydrocarbons, eg, 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 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 TF 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. 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 ECs 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 TFs activated by glucose: activator protein (AP)-2, Egr-1, upstream stimulatory factor (USF)-2, and Pax-5. This complex is different from the complex formed by AhR and AhR nuclear translocator (ARNT) (hypoxia-inducible factor [HIF]1β) 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 has been described previously.[12,15]

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Antibodies Used

Anti-AhR was from Novus Biologicals (Littleton, Colo) and Abcam (Cambridge, Mass); anti–Egr-1 was from Cell Signaling Technology (Danvers, Mass); anti–USF-1 and anti–USF-2 were from Santa Cruz Biotechnology Inc (Santa Cruz, Calif); RL2 was from Abcam; and anti–AP-2 was from AbD Serotec (Raleigh, NC).

Promoter Reporter Constructs

The fragments −280/+66 p*THBS1* and −265/+66 p*THBS1* (ΔAhR) were generated by PCR.

Mutants: (1) 5′-AGCCCGCGAGGCGA-3′; (2) 5′-AGCCCGGCTGGCGA-3′; (3) 5′-AGCCCGGCAGGCGA-3′; wild type: 5′-AGCCCGCGTGGCGCA-3′. Core sequence of the AhR binding element is underlined, and nucleotide substitutions are in bold,

Analysis of the Binding Sites for TFs in the THBS1 Promoter Region Responsive to Glucose

The sequence of p*THBS1* was analyzed using MatInspector 7.4.3 (Genomatix, http://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 TFs in glucose-stimulated human aortic ECs (HAECs) was performed using TranSignal Combo Protein/DNA array (Panomics).

Immunofluorescence

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

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

Statistical Analysis

All of the described experiments were performed more than 3 times, and the data are presented as mean values±SEM. Probability values were determined by *t* test using Microsoft Excel, and probability values of <0.05 were considered statistically significant.

Results

The Minimal Fragment of Human THBS1 Gene Responsive to High Glucose in ECs

We have reported recently that the expression of TSP-1 is increased in response to high glucose (10 to 30 mmol/L) 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 ECs and could still be 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 ECs. The −280/+66 p*THBS1* fragment was activated in response to stimulation of human umbilical vein ECs (HUVECs) by 30 mmol/L 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) (Figure 1A), suggesting that a putative binding site for the TF 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 ECs, but not in vascular smooth muscle cells[15] or mesangial cells.[41]

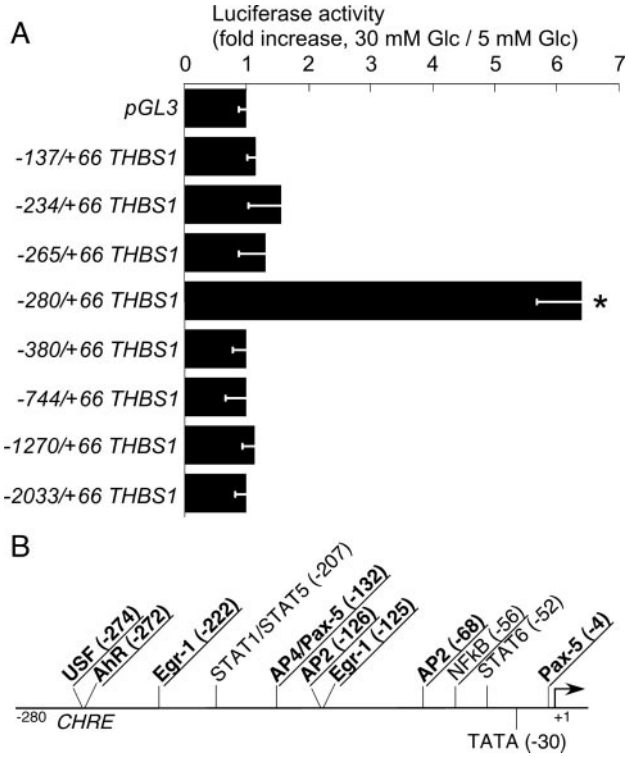

Figure 1. High glucose–responsive promoter region. A, HUVECs transfected with the promoter deletion constructs were stimulated with 30 mmol/L glucose, luciferase activity was measured 24 hours later (n=3). *$P<0.05$. B, Glucose-responsive region of the promoter was analyzed using MatInspector 7.4.3 to identify the putative binding sites for TFs. TFs activated by high glucose are underlined; TFs coprecipitating with AhR in glucose-stimulated HAECs are in bold.

We analyzed the −280/+66 fragment of *THBS1* using MatInspector (Genomatix) to identify putative binding sites for TFs. This analysis identified several putative binding sites (Figure 1B), including a binding site for AhR in the fragment responsible for glucose stimulation (−272; see Figure 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.

Identification of TFs Activated by High Glucose in ECs

We focused on 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 mmol/L glucose), we used 4 separate isolates of HAECs. A targeted proteomic approach was used to identify the activated TFs. TranSignal Protein/DNA array (Panomics Inc, Fremont, Calif) identifies only active TFs in nuclear extracts. Among the TFs activated in response to glucose treatment were six TFs whose putative binding sites have been predicted in the −280/+66 fragment of the *THBS1* promoter: AhR, AP-2, Egr-1, USF, nuclear factor (NF)-κB, and Pax-5 (Table). We have confirmed the activation of Egr-1 and AP-2 using alternative methods of detection: Northern blotting and

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Table. TFs With Putative Binding Sites in −280/+66 p*THBS1* Activated in Response to High Glucose in HAECs

Transcription Factor	Activation in Response to 30 mmol/L Glucose	*P*
AhR	8.43±2.3	0.035*
AP-2	4.11±0.57	0.025*
Egr-1	3.65±1.1	0.035*
USF	2.8±0.5	0.05*
NF-κB	3.75±0.4	0.01*
Pax-5	2.38±1.28	0.2

Data are expressed as mean fold increases±SE (n=4). *Statistically significant.

electromobility shift assay (EMSA) (see the online data supplement, available at http://circres.ahajournals.org). The values in the Table were calculated based on increase in 4 independent experiments. We have chosen to analyze only the values for TFs activated greater than 2.5-fold in at least 2 of 4 experiments. Activation of 5 of 6 TFs was statistically significant, but the values for Pax-5 did not reach statistical significance. Figure 2A demonstrates representative array results for the five TFs significantly stimulated in response to high glucose in HAECs. USF, NF-κB, 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 AP-2 by glucose.

Confirmation of Activation of AhR in Electromobility Shift Assay

Nuclear extracts from HAECs and HUVECs were used in an EMSA with a consensus AhR probe to confirm the activation

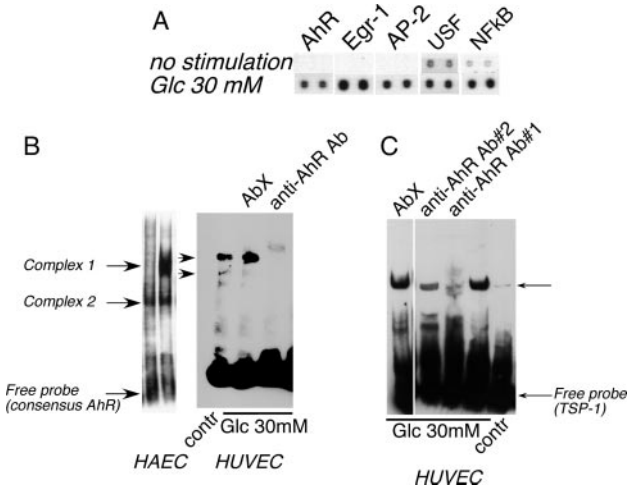

Figure 2. AhR is activated in endothelial cells in response to high glucose. A, Nuclear extracts from HAECs stimulated with glucose for 1 hour were analyzed in a Protein/DNA array (Panomics) to detect activated TFs. Representative results for AhR, Egr-1, AP-2, USF-1, and NF-κB (TFs with putative binding sites in the −280/+66 fragment of the *THBS1* promoter) are shown. B, Activation of AhR was confirmed in EMSA (5 μg 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.

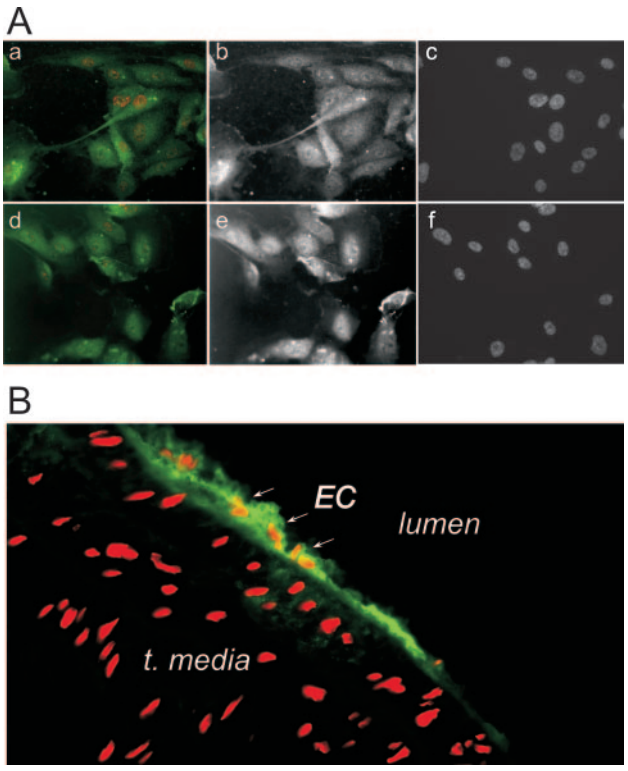

Figure 3. AhR is expressed in ECs in vitro and in vivo. Cultured HUVECs (A) and rat aorta (B) were stained using anti-AhR antibody (green). Red indicates nuclei, propidium iodide staining. Aa through Ac, unstimulated HUVECs; Ad through Af, 3 hours of 30 mmol/L glucose stimulation.

of AhR by high glucose (Figure 2B). Two DNA-binding protein complexes in both EC types were activated in response to 30 mmol/L glucose (complex 1 and complex 2; Figure 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 (Figure 2C).

AhR Is Expressed in ECs

AhR is constitutively present in the cytosol and is translocated to the nucleus on activation by its ligand.[44–46] We have detected AhR in both cultured HAECs and luminal ECs of the rat aorta (Figure 3A and 3B).

AhR Associates With TSP-1 Promoter in Glucose-Stimulated HAECs

Chromatin immunoprecipitation from HAECs stimulated with 30 mmol/L 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 on glucose treatment, whereas the general nuclear factor TFII is associated with the promoter in both control and glucose-treated ECs (Figure 4A).

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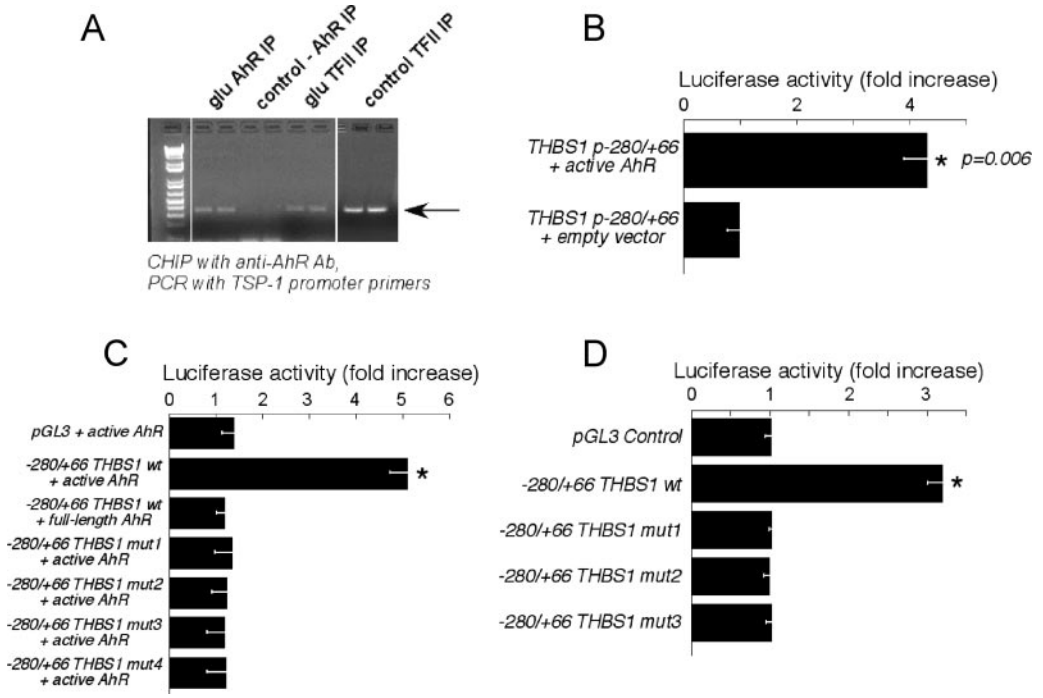

Figure 4. Active AhR binds and activates *THBS1* promoter. A, HAECs (1-hour glucose) were used for chromatin immunoprecipitation with anti-AhR antibody. DNA from the precipitated fraction was used in PCR to amplify the *THBS1* promoter fragment. B, HEK293 were cotransfected with the −280/+66 *THBS1* fragment reporter plasmid and cDNA of constitutively active AhR; luciferase activity was measured 24 hours later (n=3). **P*<0.05. C, HEK293 were cotransfected with indicated combinations of plasmids, and the activity of luciferase was measured 24 hours later. Increase in the luciferase activity of the control, wild-type −280/+66, or mutant −280/+66 promoter constructs cotransfected with active or full-length AhR over the activity of the same promoter cotransfected with empty pcDNA3 is shown on the graph (n=3). *P*<0.05. D, HUVECs were transfected with the wild-type −280/+66 promoter fragment or mutant fragments and stimulated with 30 mmol/L glucose for 24 hours; ratio of the luciferase activity in glucose-stimulated cells to nonstimulated cells (n=3). **P*<0.05.

Constitutively Active AhR Activates the THBS1 Promoter

When the constitutively active form of AhR was cotransfected 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 with cells transfected with the control plasmid (Figure 4B). The full-length AhR (inactive form) failed to induce any stimulation of the promoter (Figure 4C).

To confirm that the promoter activity in response to active AhR is dependent on the binding site for this TF (−272), we generated mutant −280/+66 p*THBS1* promoter constructs with 1, 2, and 3 nucleotide substitutions in the core of the AhR binding site as described in Materials and Methods (Mut1, -2, and -3). The activity of these mutants remained at the basal level when cotransfected with active AhR (Figure 4C). When HUVECs 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 (Figure 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 ECs

Several TFs are known to form complexes with active AhR. The most common is HIF1β (ARNT), which associates with AhR in response to xenobiotic activators (reviewed elsewhere[47]); other proteins, including TFs, have been identified (reviewed elsewhere[45,48]). We have used a TF/TF interaction array (Panomics) that detects only active TFs in complex with the TF of interest and is based on the immunoprecipitation. The experiments revealed that AhR can be precipitated in complex with several TFs 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 AP-2, Egr-1, USF, and Pax-5 (Figure 5A), suggesting that these TFs 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 nonimmune mouse IgG (data not shown).

To confirm these TFs form a complex in response to high glucose, we used corresponding antibodies in the binding reaction with consensus AhR probe (Figure 5B and 5C) and TSP-1 probe (Figure 5D). Formation of the specific complexes was prevented by anti-AhR antibody, whereas an unrelated antibody did not affect the binding (Figure 5B). To characterize the complex of TFs, the following antibodies were used in combination with the AhR consensus probe in EMSA: anti-AhR (Figure 2B and 2C and Figure 5B through 5D), anti–Egr-1 (Figure 5B through 5D), anti–AP-2 (Figure 5C and 5D), anti–USF-1, and anti–USF-2 (Figure 5C and 5D). With the exception of USF-1, all of these TFs were found in complex with AhR. Anti-AhR, anti–Egr-1, anti–AP-2, 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 TFs (Figure 5B).

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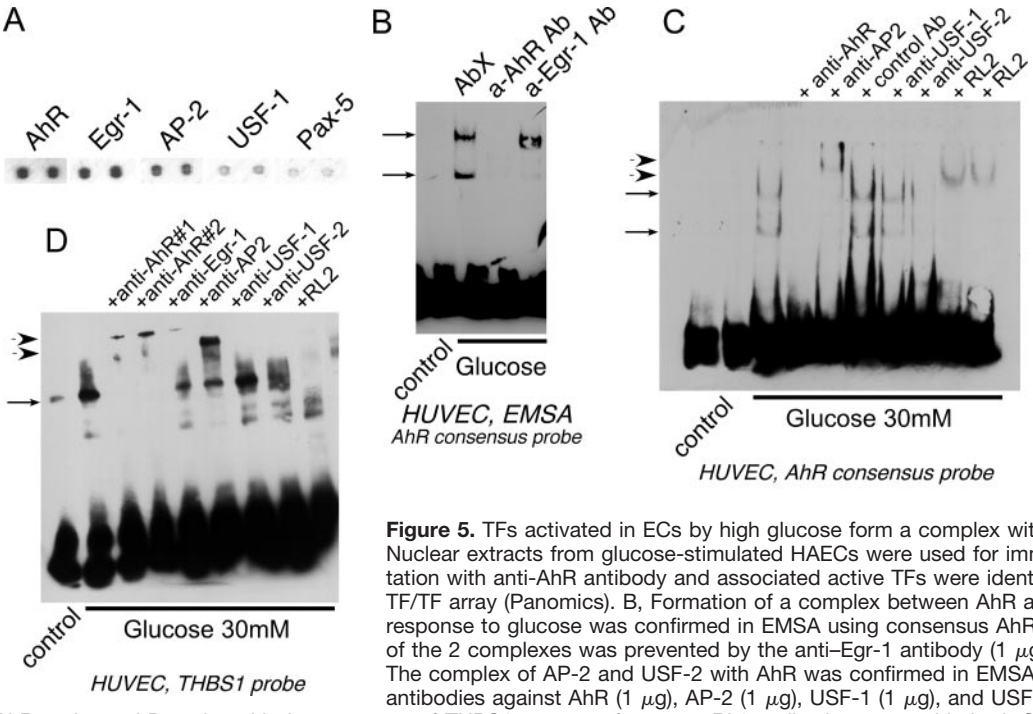

Figure 5. TFs activated in ECs by high glucose form a complex with AhR. A, Nuclear extracts from glucose-stimulated HAECs were used for immunoprecipitation with anti-AhR antibody and associated active TFs were identified using TF/TF array (Panomics). B, Formation of a complex between AhR and Egr-1 in response to glucose was confirmed in EMSA using consensus AhR probe. One of the 2 complexes was prevented by the anti–Egr-1 antibody (1 μg). C and 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); and C-consensus AhR probe and D-probe with the sequence of *THBS1* promoter fragment. RL2 antibody was used in both C and D to prove that at least 1 of the proteins is O-glycosylated.

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 (data not shown). Antibody against HIF1β, the most common partner of AhR, did not affect either of the 2 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 (Figure 5C and 5D). To confirm that glycosylation regulates the formation of the complex or its DNA-binding activity, we treated ECs with: (1) 6-diaz-*O*-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 (Figure 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 ECs 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 deglycosylation, and a certain level of glycosylation is required 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

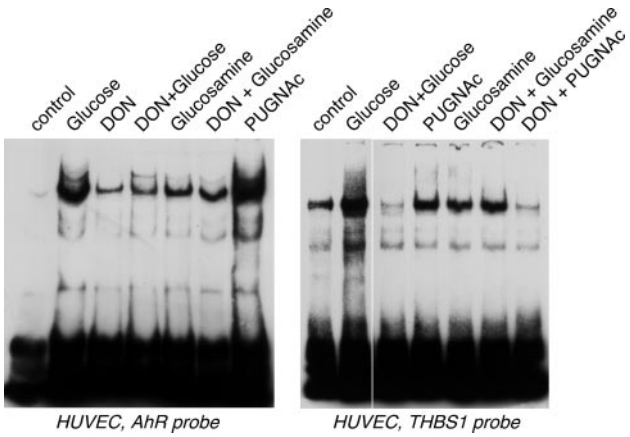

Figure 6. Glycosylation regulates the activity of the DNA-binding complex. A total of 5 μg nuclear extracts from HUVECs treated for 24 hours with 30 mmol/L glucose, 1 mmol/L glucosamine, 100 μmol/L DON, or 40 μmol/L PUGNAc was used in EMSA with the consensus AhR probe (left) or TSP-1 probe (right).

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of activation remain 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 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 ECs and that AhR controls the expression of TSP-1, a potent antiangiogenic and proatherogenic 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, which 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. The regulation of the *THBS1* promoter by high glucose in ECs 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 ECs 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, 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, coactivators, and suppressors. TFs and coactivators form signal- and cell type–specific multiprotein complexes on the promoters. We have identified 5 proteins in complex with AhR in glucose-stimulated ECs: AP-2, Egr-1, USF-2, and Pax-5. Interestingly, but not surprisingly, the putative binding sites for all 4 TFs were predicted in the glucose-responsive fragment of the *THBS1* promoter in close proximity to the AhR binding site (MatInspector). Of the multiple TFs with predicted binding sites in this region, only 5 were consistently activated (at least 2.5-fold) in response to high glucose in HAECs (AhR, AP-2, Egr-1, USF, NF-κB), and 4 of them represented the proteins found in complex with AhR in both coprecipitation and supershift experiments with the corresponding antibodies. Although the probe-based analyses did not allow to distinguish between USF-1 and USF-2, which 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. An anti–USF-1 antibody that we used to supershift USF-1 complex in an unrelated study with a different cell type (data not shown) failed to change the binding of the AhR complex to both AhR concensus and AhR *THBS1* probe.

Although the activation of Egr-1 and NF-κB in ECs and the activation of USF in other cell types and tissues in response to hyperglycemia or in diabetics has been reported previously,[42,50–53] this is the first report of activation of AhR and AP-2 by high glucose. An antibody against HIF1β did not affect AhR complexes in EMSA, and this TF was not activated by high glucose in ECs, suggesting that AhR-dependent transcriptional mechanisms activated in response to glucose differ from the well-described mechanisms activated by xenobiotics.

Our data clearly demonstrate that activation of the transcriptional complex depends on glycosylation of at least 1 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, whereas the inhibitors of deglycosylation 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 specificity protein (SP)-1 in response to stimulation with high glucose was previously reported in both ECs[54] and vascular smooth muscle cells.[55] SP1 undergoes posttranslational 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 ECs or in vascular smooth muscle cells (data 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 TFs 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 ECs to hyperglycemia. Despite the rapidly accumulating evidence that ECs 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 antiangiogenic and proatherogenic protein

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involved in the development of multiple diabetic vascular complications.

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Disclosures

None.

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Aryl Hydrocarbon Receptor Is Activated by Glucose and Regulates the Thrombospondin-1 Gene Promoter in Endothelial Cells

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Materials and Methods

Cell culture and stimulation with high glucose: Human aortic EC (HAEC) were purchased from Cambrex (East Rutherford, NJ), HEK-293 from ATCC (Manassas, VA). Human umbilical vein EC (HUVEC) were kindly provided by Dr. Paul DiCorleto (Cleveland Clinic). Primary EC with passage numbers between 3 and 12 were used. Cells were stimulated with glucose as described previously[1, 2].

Antibodies: Antibodies against AhR were from Novus Biologicals (Littleton, CO) and Abcam (Cambridge, MA) (RPT1 and RPT9). Anti-Egr-1 antibody was from Cell Signaling Technology (Danvers, MA), anti-USF-1 and anti-USF-2 were 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 p*THBS1* and -265/+66 p*THBS1* (ΔAhR) were generated from -2033/+66 p*THBS1* (a kind gift from Dr. Bornstein, University of Washington, Seattle) by PCR using the primers 5'CGAGCCCGCGTGGCGCAAGAG and 5'CAAGAGTACGAGCGCCGAGCCCG respectively in combination with 5'TCCGGAGTAGAGGTTGCTCCTGG and cloned into basic pGL3 (Promega, Madison, WI).

The mutants of -280/+66 p*THBS1* were designed based on the decoy oligonucleotide experiment (data not shown): several oligonucleotides with mutations in the binding site for AhR were tested in a co-transfection experiment to identify the ones that did not prevent activation of -280/+66 by glucose. Mutant 1 has the sequence

5'AGCCCGCG**A**GGCGA3', mutant 2 – 5'AGCCCG**GC**TGGCGA3', and mutant 3 – 5'AGCCCG**GCA**GGCGA3' (wt sequence is

5' AGCCCGCGTGGCGCA 3'). Mutation were introduced using the QuickChange Lightning Site-Direted Mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. The primers used to generate the mutants were 1) forward: 5' CACCCCGAGCCCGCGAGGCGCAAGAGTACGAGCGCCGAG; reverse: 5' CTCGGCGCTCGTACTCTTGCGCCTCGCGGGCTCGGGGTG; 2) forward: 5' CACCCCGAGCCCGGCTGGCGCAAGAGTACGAGCGCCGAG; reverse: 5' CTCGGCGCTCGTACTCTTGCGCCAGCCGGGCTCGGGGTG; 3) forward: 5' CACCCCGAGCCCGGCAGGCGCAAGAGTACGAGCGCCGAG; reverse: 5' CTCGGCGCTCGTACTCTTGCGCCTGCCGGGGCTCGGGGTG.

Analysis of the binding sites for transcription factors in the THBS1 promoter region responsive to glucose: The sequence of p*THBS1* was analyzed using MatInspector 7.4.3 (Genomatix, www.genomatix.de). The program uses matrices and algorithms described in [3].

Gel shift assay (EMSA): Nuclear extracts were prepared using a Nuclear Extraction kit (Panomics, Fremont, CA) as per manufacturer instructions. The sequences of probes used were as follows:

AhR consensus – 5'GGGGATCGCGTGACAACCC,

TSP-1 – 5'TCACCCCGAGCCCGCGTGGCG,

Egr-1 – 5'GGATCCAGCGGGGGCGAGCGGGGGCCA,

AP2 – 5'GATCGAACTGACCGCCCGCGGCCCGT

Plasmids for the expression of AhR: Full-length AhR cDNA was obtained from ORIGENE (Rockville, MD). ORF was obtained by PCR using the commercial plasmid as a template and the primers 5'-GCGGCCGCACCACTAAGGACTAAAAATG and 5'-TGCGGCCGCTAGTTTGTGTTTGGTTCTA and cloned into pcDNA3. The constitutively active form of AhR was prepared by constructing the AhR deletion mutant as described previously for murine AhR[4].

Cell transfections and Luciferase Reporter Assay: The transfection procedure was carried out using Lipofectin reagent (Invitrogen) following the manufacturer's protocol, and the transfected cells were treated with 30 mM glucose. Cell extracts were assayed for luciferase activity using a Luciferase assay kit (Promega). The activity of luciferase was normalized to protein concentrations in lysates.

Chromatin Immunoprecipitation: Chromatin immunoprecipitation assays were performed using ChIP kit as per the manufacturer's protocol (Active Motif, Carlsbad, CA). Results were analyzed by PCR with primers designed to amplify the 180-bp region of the TSP-1 promoter containing the putative binding site for AhR predicted using MatInspector7.4.3. (5'-TTTCTCTATCGATAGGTACCGAGCTC-3' and 5'-CCCGGGAGTAGAGGTTGCTCCTGGA-3').

Analysis of activation of transcription factors in glucose-stimulated HAEC: Nuclear extracts were subjected to the TranSignal Combo Protein/DNA array (Panomics) according to the manufacturer's instructions. Quantification of signals was done using Photoshop (Adobe, San Jose, CA).

Immunofluorescence: Anti-AhR primary antibody (Novus Biologicals)(1:50 in blocking solution). and goat anti-mouse Alexa Fluor-labeled secondary antibody (Invitrogen) (1:1000 in blocking solution) were used.

Sections of rat aorta were prepared as described earlier[1] from control lean Zucker rats and stained with the anti-AhR antibody as described above.

Treatment of EC with glycosylation inhibitors and metabolites of hexosamine pathway was done as described earlier[2].

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.

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Online Figure I. Confirmation of activation of Egr-1 and AP-2 by high glucose in EC. A: Increase in Egr-1 mRNA was detected in 30 mM glucose-stimulated HUVEC, Northern blotting. **B:** Activation of Egr-1 in HUVEC by high glucose was confirmed in EMSA with Egr-1 consensus probe. Specificity of DNA-binding complex was confirmed in competition by a cold Egr-1 probe and inhibition of the complex formation with anti-Egr-1 antibody (1 μg per reaction). **C:** AP-2 activation by high glucose in HAEC was confirmed in EMSA using radiolabeled consensus AP-2 probe. Cold AP-2 probe, but not the control cold probe, competed with the two complexes formed in response to high glucose.

Online Figure I