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.

References:

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3. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics.* 2005;21:2933-2942.

4. McGuire J, Okamoto K, Whitelaw ML, Tanaka H, Poellinger L. Definition of a dioxin receptor mutant that is a constitutive activator of transcription: delineation of overlapping repression and ligand binding functions within the PAS domain. *J Biol Chem.* 2001;276:41841-41849.

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