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Upstream Stimulatory Factor-2 Mediates Quercetin-Induced Suppression of PAI-1 Gene Expression in Human Endothelial Cells

Nélida C. Olave[1], **Maximiliano H. Grenett**[1], **Martin Cadeiras**[1], **Hernan E. Grenett**[1], and **Paul J. Higgins**[2,*]

[1]Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

[2]Center for Cell Biology and Cancer Research, Albany Medical College, Albany, New York

Abstract

The polyphenol quercetin (Quer) represses expression of the cardiovascular disease risk factor plasminogen activator inhibitor-1 (PAI-1) in cultured endothelial cells (ECs). Transfection of PAI-1 promoter-luciferase reporter deletion constructs identified a 251-bp fragment (nucleotides −800 to −549) responsive to Quer. Two E-box motifs (CACGTG), at map positions −691 (E-box1) and −575 (E-box2), are platforms for occupancy by several members of the c-MYC family of basic helix-loop-helix leucine zipper (bHLH-LZ) proteins. Promoter truncation and electrophoretic mobility shift/supershift analyses identified upstream stimulatory factor (USF)-1 and USF-2 as E-box1/E-box2 binding factors. ECs co-transfected with a 251 bp PAI-1 promoter fragment containing the two E-box motifs (p251/luc) and a USF-2 expression vector (pUSF-2/pcDNA) exhibited reduced luciferase activity versus p251/luc alone. Overexpression of USF-2 decreased, while transfection of a dominant-negative USF construct increased, EC growth consistent with the known anti-proliferative properties of USF proteins. Quer-induced decreases in PAI-1 expression and reduced cell proliferation may contribute, at least in part, to the cardioprotective benefit associated with daily intake of polyphenols.

Keywords

PAI-1; ENDOTHELIAL CELLS; TRANSFECTION; PROLIFERATION; TRANSCRIPTION FACTOR; USF

Polyphenols are abundant dietary micronutrients with a potential wide range of beneficial effects on human health. Epidemiologic data indicate, in fact, that the intake of polyphenols, such as quercetin (Quer), are inversely associated with mortality from cardiovascular disease (CVD) [Hertog et al., 1993; Knekt et al., 2001]. Quer is a potent anti-oxidant and metal ion chelator with anti-inflammatory, anti-neoplastic, and cardio-protective activities [Fresco et al., 2006]. This polyphenol inhibits the proliferation and migration of human aortic smooth muscle cells [Alcocer et al., 2002] likely through the transcriptional regulation of relevant genes [Abou-Agag et al., 2001; Pan et al., 2008] among the most prominent of which is plasminogen activator inhibitor-1 (PAI-1), the major regulator of pericellular plasmin generation [Pasten et al., 2007]. Elevated PAI-1 expression is an important pathologic contributor to the development and progression of CVD leading to neointimal disease,

*Correspondence to: Dr. Paul J. Higgins, PhD, Center for Cell Biology and Cancer Research, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208. higginp@mail.amc.edu.

thrombosis formation, perivascular fibrosis, and eventual myocardial infarction [DeYoung et al., 2001; Kaikita et al., 2001; Pandolfi et al., 2001; Wu and Zhao, 2002; Chen et al., 2006; Westrick and Eitzman, 2007; Alessi and Juhan-Vague, 2008; Samarakoon and Higgins, 2008].

Among the PAI-1 transcriptional control elements implicated in CVD risk is the hexanucleotide E-box motif CANNTG that is a recognition platform for several members of the MYC family of the bHLH-LZ transcription factors including c-MYC, upstream stimulatory factors 1 and 2 (USF-1/2) as well as hypoxia-inducible factor-α (HIF-α) [Sawadogo and Roeder, 1985; Samoylenko et al., 2001]. The USF-1 and USF-2 genes encode the 43 and 44 kDa USF proteins 1 and 2, respectively, that function as both homo- and heterodimers to impact transcriptional outcomes [e.g., Corre and Galibert, 2005; Qi et al., 2006]. USF-1 and USF-2 have identical DNA binding specificities and, although their transcriptional activities are often gene-dependent, distinct cell-type ratios of USF homodimers and heterodimers are common which appears important in gene regulation [Sirito et al., 1994; Viollet et al., 1996; Massari and Murre, 2000; Dimova and Kietzmann, 2006]. USF-mediated PAI-1 transcriptional controls, however, may depend on cell-type rather than promoter context requiring confirmation of target gene activity in any specific cell system [e.g., Dimova and Kietzmann, 2006].

In this article we report that Quer stimulates binding of USF-2 to two distinct E-box sequences in the PAI-1 promoter resulting in down-regulation of PAI-1 expression in human coronary artery endothelial cells (HCAECs). Consistent with the anti-proliferative effects of USF proteins in other cell systems [Ismail et al., 1999; Qyang et al., 1999; Szentirmay et al., 2003; Corre and Galibert, 2005; Jung et al., 2007], transfection of human microvessel ECs with a dominant-negative (DN) USF construct stimulated, while USF-2 overexpression inhibited, cell growth. USF-2 clearly affects both the proliferative and proteolytic programs in human ECs and may, therefore, be an important contributor to the cardiovascular benefits of quercetin exposure.

MATERIALS AND METHODS

CELL CULTURE

Primary HCAECs (Cambrex, Walkersville, MD) were used between the 6th and 8th passage [Pasten et al., 2007]. Human microvessel endothelial (HMEC-1) cells were grown in DMEM/10% FBS/10 ng/ml EGF. Opti-MEM1 reduced serum medium was from BRL. Quer (Sigma) concentrations are indicated in the text.

pPAI-251/LUC, pE-BOX1/LUC, AND pE-BOX2/LUC CONSTRUCTS

The PAI-1 promoter reporter constructs, pPAI-800/luc, pPAI-549/luc, pPAI-308/luc, and pPAI-251/luc were described previously [Grenett et al., 1998a, 2001]. To generate the pE-box1/luc and pE-box2/luc constructs, complementary oligonucleotides containing three tandem E-box1 sequences (Forward: 5′-CGTCTGGACACGTGGGGGAGTGTCTGGACACGTGGGGGAGTGTCTGGACACGTGGGGGAGT-3′; Reverse: 5′-CTCGAGACTCCCCCACGTGTCCAGACACTCCCCCACGTGTCCAGACACTCCCCCACGTGTCCAGAGCT-3′) or three tandem E-box2 sequences (Forward: 5′-CGACAATCACGTGGCTGGCTAGACAATCACGTGGCTGGCTAGACAATCACGTGGCTGGCTAC-3′; Reverse: 5′-CTCGTAGCCAGCCACGTGATTGTCTAGCCAGCCACGTGATTGTCTAGCCAGCCACGTGATTGTCAGCT-3′) were synthesized with *Sca*I/*Xho*I restriction sites (underlined), annealed, and subcloned into the *Sca*I/*Xho*I site of the pGL3-control vector. Sequencing and

restriction enzyme mapping verified all constructs. The pUSF-2/pcDNA3 expression vector was a kind gift of Dr. Benoit Viollet (Institut Cochin, University of Paris).

ENDOTHELIAL CELL TRANSFECTION

DNA-Lipofectin complexes were added to semi-confluent cultures in 12-well multiwell plates in Opti-MEM-1 for 5 h at 37°C [Grenett et al., 1998b, 2000]. After transfection, cells were rinsed, incubated in complete media for 18 h then stimulated with Quer (10 μmol/L) for 6 h at 37°C in serum-free medium. All constructs were co-transfected with a thymidine kinase promoter-driven *Renilla* luciferase reporter (pRL-TK) [Farr and Roman, 1992; Lorenz et al., 1996]. Firefly luciferase activities were normalized to corresponding *Renilla* luciferase signal to correct for variations in DNA uptake when using different plasmids.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Nuclear extracts [Grenett et al., 2001] were incubated with ^{32}P-labeled E-box1 or E-box2 probes. Nonlabeled wild-type or mutant deoxyoligonucleotides were added at 100-fold molar excess prior to addition of the ^{32}P-labeled target sequences (Table I). Supershift antibodies were to the N-terminus of USF-1 or USF-2 (Santa Cruz). For certain analyses, nuclear extracts were treated with lambda protein phosphatase. Dried gels were analyzed with a Molecular Dynamics PhosphoImager (Molecular Dynamics).

PROLIFERATION ASSAY

Cells were cultured in 24-well plates at a density of 15 ×10^3/well, growth arrested in medium containing 0.5% calf serum for 24 h, incubated with or without Quer (10 μmol/L), or transfected with the pUSF-2/pcDNA [Fresco et al., 2006] construct or empty vector prior to maintenance for 0, 24, 48, and 72 h in media containing 5% calf serum. Cell proliferation was determined using the CyQUANT assay (Invitrogen). Briefly, CyQuant GR solution (200 μl) was added to each well, incubated for 5 min at room temperature, relative fluorescence (480-nm excitation, 520-nm emission) measured with a Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, VT) and standardized for cell number. HMEC-1 cells were transfected with a DN-USF (USF-A) construct [Allen et al., 2005], maintained in complete HMEC-1 growth medium and the fraction of Ki-67-expressing cells assessed immunocytochemically.

STATISTICAL ANALYSIS

All data were evaluated using the Student's *t*-test; values of $P < 0.05$ were considered significant.

RESULTS

EFFECT OF QUER ON PAI-1 PROMOTER ACTIVITY

We demonstrated previously that Quer down-regulates PAI-1 expression in cultured HCAECs likely through responsive sequences in a 1.1 kb promoter region (pPAI-1100/luc construct) [Pasten et al., 2007]. To further define the molecular basis of PAI-1 gene regulation, ECs transiently transfected with 5′ deletion constructs of the pPAI-1100/luc were incubated in the absence or presence of 10 μmol/L Quer for 6 h. pPAI-800/luc reporter activity declined by ~55% in Quer-treated cells; pPAI-549/luc and pPAI-308/luc signals, in contrast, were unaffected (Fig. 1) suggesting that Quer-responsive promoter elements likely reside in the 251-bp promoter fragment upstream of bp −549 (i.e., bps −800 to −549) relative to the start site of PAI-1 transcription. This 251-bp region had significant independent functional potential since ECs transfected with the pPAI-251/luc reporter and

stimulated with Quer exhibited the same ~50–60% reduction in luciferase activity compared with control as did the pPAI-800/luc construct (Fig. 2).

FUNCTIONAL ANALYSIS OF THE PAI-1 E-BOX1 AND E-BOX2 SITES

The 251-bp PAI-1 promoter fragment contains two E-box motifs (CACGTG) that map to positions −691 (E-box1) and −575 (E-box2) (Fig. 3). These hexanucleotide sequences are recognized binding platforms for bHLH-LZ proteins of the c-MYC family. E-box2, moreover, is a typical classic "B" class sequence as the CACGTG motif is preceded by a T residue (Table I). EC cells transfected with luciferase reporters driven by tandomly generated triplicate sequences representing either E-box1 or E-box2 were incubated in the absence or presence of Quer (10 μmol/L) to determine the independent functionality of E-box1 and E-box2. Quer-treated transfectants (both E-box1 and E-box2) exhibited the typical 40–50% reduction in normalized luciferase activity compared to untreated controls (Fig. 4).

Since E-box motifs are functionally significant in the response to Quer, nuclear extracts from Quer-treated or -untreated ECs were incubated with ^{32}P-labeled double-stranded deoxyoligonucleotides spanning E-box1 and E-box2 and their respective flanking sequences to identify binding elements (Table I). Extracts from Quer-treated cells had enhanced binding to both E-box target probes compared to untreated controls (Fig. 5A,B). Binding specificity was confirmed by addition of a 100× molar excess of unlabeled competitor wild-type sequences or the appropriate mutants. As a first approximation to identify the potential binding factors, antibodies against USF-1, USF-2, or c-MYC were included in the EMSA reaction. Addition of either USF-1 or USF-2 (but not c-MYC) antibodies supershifted the DNA–protein complexes (Fig. 5) while pre-incubation of Quer-treated nuclear extracts with lambda phosphatase prior to addition of labeled probe effectively inhibited the formation of the typical band shift pattern, consistent with the phosphorylation requirements of USF proteins for probe recognition.

The most prominent supershift was generated using USF-2 antibodies (Fig. 5A,B) and whereas the transcriptional activities of USF family members often gene-specific [Dimova and Kietzmann, 2006], it was important to determine if USF-2 represses luciferase activity of the p251/luc construct. Cells were co-transfected with p251/luc and a plasmid encoding the USF-2 transcription factor (pUSF-2/pcDNA3) and luceriferase reporter signal assessed as described in Figure 1. Co-transfectants had a ~70–80% reduction in luciferase activity compared with ECs transfected with the p251/luc alone (Fig. 6).

EFFECT OF QUER AND USF-2 ON ENDOTHELIAL CELL PROLIFERATION

Quer inhibits proliferation in both human vascular smooth muscle cells and microvascular ECs [Alcocer et al., 2002; Fan et al., 2003]. PAI-1 is differentially associated with both cellular proliferation as well as the onset of replicative senescence [e.g., Kortlever et al., 2006; Qi et al., 2006] likely as a consequence of the relative level of expression of this SERPIN in specific cell types. USF family members also regulate an anti-proliferative program by initiating a cell-cycle stage-specific arrest [Jung et al., 2007]. Since Quer represses PAI-1 expression through USF-2-binding E-box sites and overexpression of USF-2 suppresses activation of a PAI-1 promoter-dependent reporter (Fig. 6), we examined the effect of Quer and USF-2 on cell proliferation as well as the effect of inhibition of USF function on HMEC-1 growth. ECs exposed to Quer, or transfected with pUSF-2/pcDNA, showed a time-dependent proliferative arrest compared with untreated cells or empty vector transfectants (Fig. 7A). Conversely, expression of the DN-USF construct (USF-A) markedly increased the number of Ki-67-positve HMEC-1 cells (Fig. 7B) indicating that manipulation of the level or function of these c-MYC family members has profound consequences on EC growth (Fig. 7B).

DISCUSSION

The polyphenols catechin and quercetin transcriptionally repress PAI-1 gene expression in cultured HCAEC [Pasten et al., 2007]; the 251-bp fragment that maps to nt −800 and −549 in the PAI-1 promoter likely mediates Quer-initiated PAI-1 repression. Two resident E-box motifs (CACGTG), at positions −691 (E-box1) and position −575 (E-box2), are binding platforms for bHLH-LZ transcription factors including members of the MYC family (e.g., USF-1/2, c-Myc/Max). Functional analysis of this pPAI-251/luc fragment confirmed that Quer treatment effectively suppressed p251luc reporter activity. pE-box1/luc or pE-box2/luc constructs, moreover, independently exhibited Quer-dependent transcriptional inhibition implicating both E-box motifs as functional sites in Quer-mediated PAI-1 repression in human ECs.

Gel shift/competition analyses using labeled E-Box1 or E-Box2 oligonucleotide probes further indicated that intact E-box consensus motifs are necessary for site occupancy by USF-1/2 dimers. It appears, however, that the two USF species differ in affinity for, or relative abundance on, the two PAI-1 E box sites reflecting, perhaps, chromatin context, particular flanking DNA sequences or the low abundance of USF-1 in ECs but also cooperative or indirect binding with other accessory co-repressors. Although these PAI-1 E-Box motifs can, theoretically, bind both USF and c-MYC, c-MYC does not occupy the PAI-1 promoter in Quer-treated ECs likely because B-class E boxes are, at least in certain genes, preferential USF targets. Overexpression of wild-type or DN forms of USF or c-MYC revealed selectivity in gene activation by USF or c-MYC, highlighting the importance role of promoter context in determining whether a given E-box is selectively regulated by USF and c-MYC [Bruno et al., 2004]. Nuclear extracts treated with phosphatase before the binding assays, moreover, confirmed that USF phosphorylation is required for PAI-1 E-box motif occupancy in ECs as is the case in human keratinocytes [e.g., Qi et al., 2006], consistent with the phosphorylation requirement of certain bHLH-LZ factors for E-Box motif recognition [Cheung et al., 1999; Providence et al., 2002; Ma et al., 2007]. Finally, the role of USF-2 in PAI-1 repression of PAI-1 was confirmed by co-transfection of ECs with p251/luc and USF-2/pcDNA constructs, illustrating that USF-2-dependent expression controls may reflect both cell type [e.g., Qi et al., 2006; this article] and Quer- or growth factor-related recruitment of co-repressors or co-activators, respectively.

Given the central role of USF-2 in growth control, it was necessary to determine whether increased expression of USF-2 affected vascular cell proliferation. Cell proliferation was significantly decreased in cells treated with Quer or transfected with USF-2 and stimulated almost threefold in ECs expressing a DN-USF construct. USF overexpression inhibits cell proliferation in papillary thyroid cancer cells line and rat embryo fibroblasts among others [Aperlo et al., 1996; Luo and Sawadogo, 1996; Qyang et al., 1999; Jung et al., 2007] and loss of USF transcriptional activity is a frequent occurrence in breast cancer [Ismail et al., 1999]. The anti-proliferative effect of USF, however, may not be common to most cells types. In Saos-2 osteosarcoma, for example, USF did not inhibit proliferation [Qyang et al., 1999]. Thus, the transcriptional activity of USF proteins appear to be context dependent and may indicate that modulation of USF action by transcriptional co-activators and post-translational modification may be cell-type specific. While the exact mechanism responsible for suppression of cellular proliferation is not clear, USF might exert an inhibitory effect through the activation of cell-cycle regulatory proteins [Corre and Galibert, 2005; Jung et al., 2007]. Collectively, the present findings suggest that USF-2 is an important regulator of both PAI-1 expression and proliferation by Quer in ECs. These results may provide both a rationale for the potential use of quercetin in the prophylaxis of CVDs and insights as to the molecular basis for such novel therapies.

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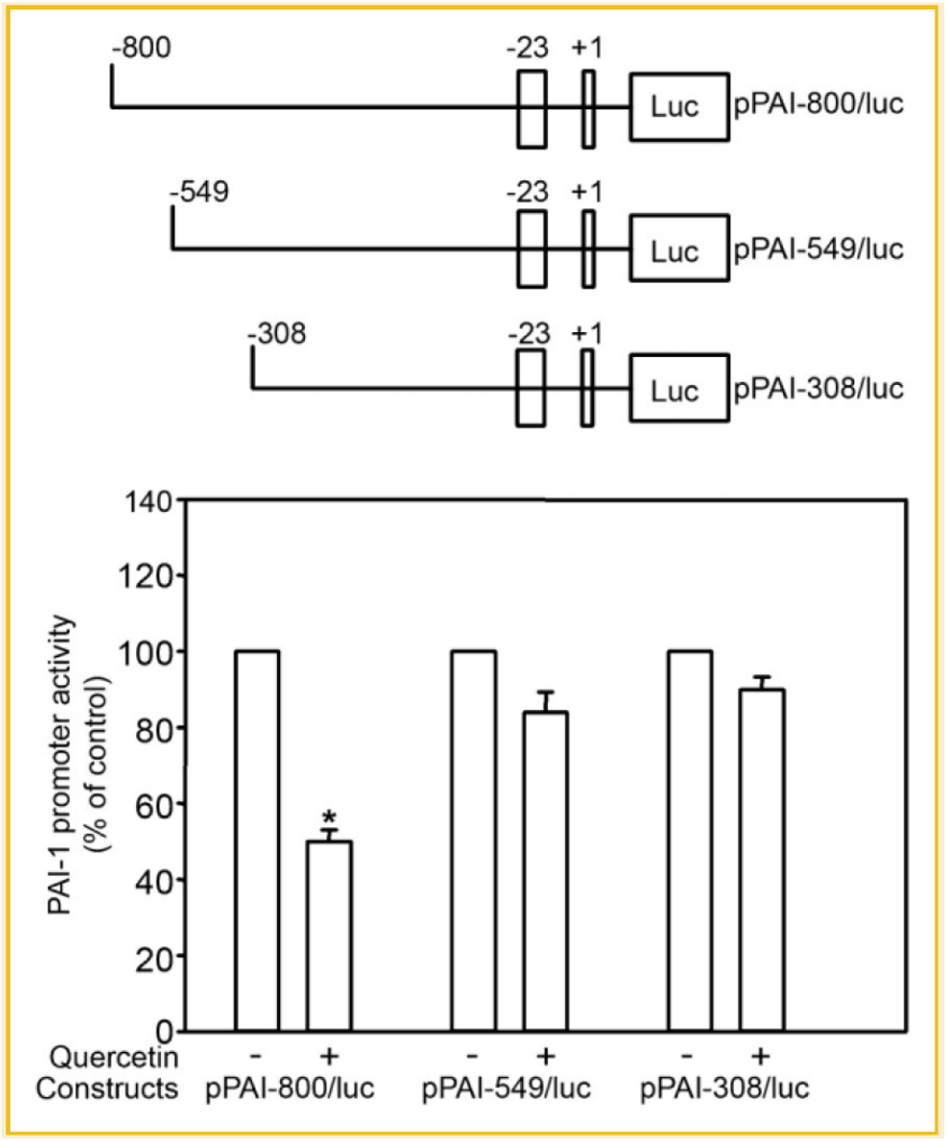

Fig. 1.

Identification of a quercetin responsive region between nt −800 and −549 in the PAI-1 promoter. A: Schematic representation of the luciferase reporter constructs containing the pPAI-800/luc, pPAI-549/luc, and pPAI-308/luc) of human PAI-1 promoter. B: ECs transiently transfected with pPAI-800/luc, pPAI-549/luc, and pPAI-308/luc were treated with quercetin or vehicle for 6 h. The columns and vertical bar represent means ± SE of five separated replicates; $*P \leq 0.05$.

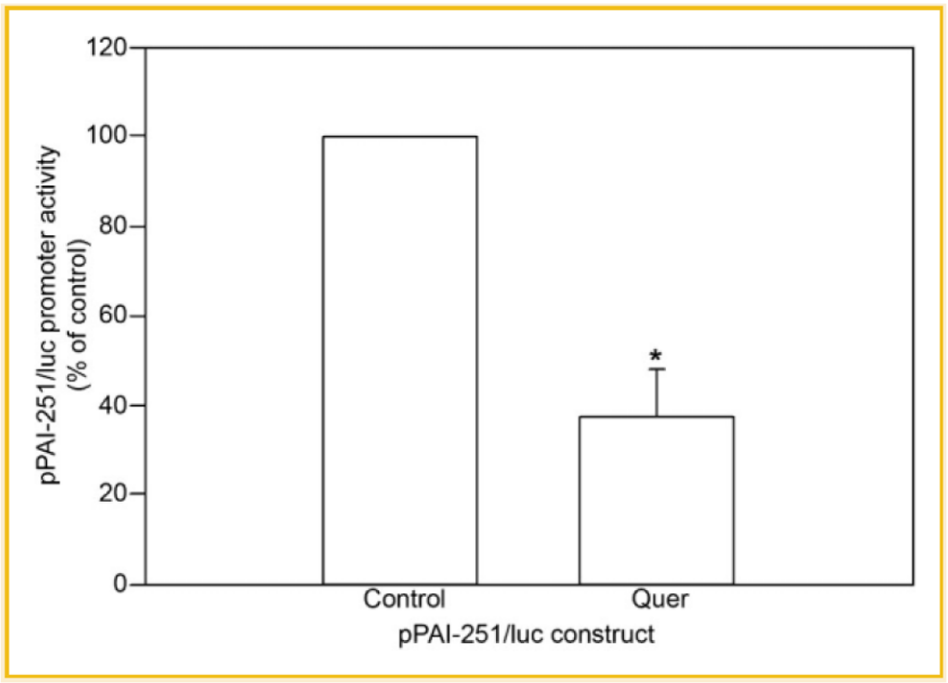

Fig. 2.

Functional analysis of the 251-bp promoter fragment of the PAI-1 gene. ECs were transfected with the pPAI-251/luc reporter construct and treated or not with 10 μM quercetin. The relative luciferase activity of unstimulated HCAEC was set as 100%. Values were expressed as mean ± SE of five individual experiments; $*P \leq 0.05$.

Fig. 3.

The human PAI-1 gene promoter region spanning nucleotides −813 to −563. Position of the E-box1 (−691) and E-box2 (−575) are represented in boldface.

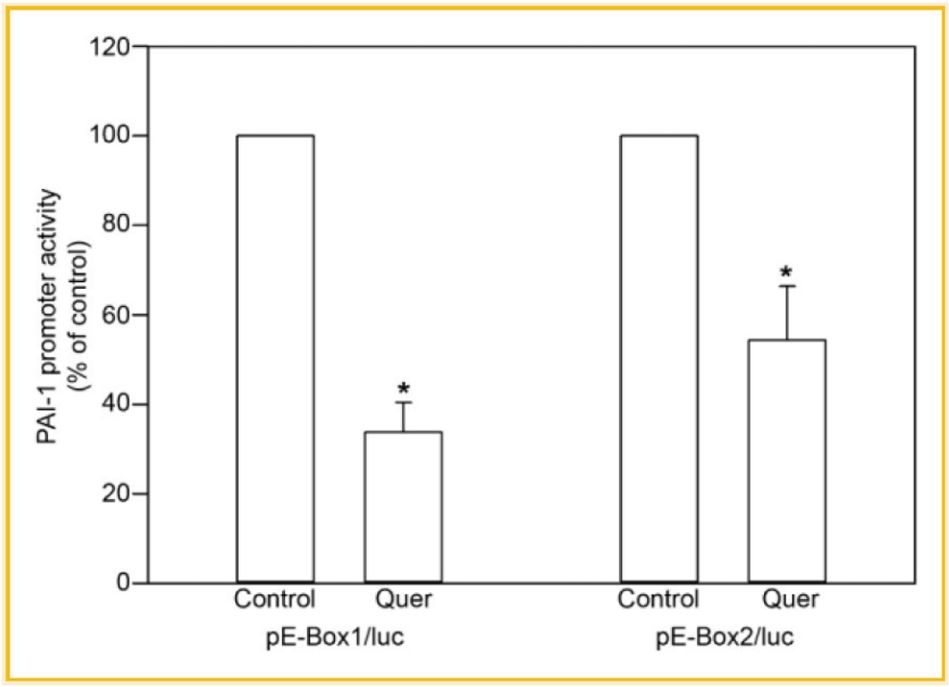

Fig. 4.

Functional analysis of the PAI-1 E-box1 and E-box2. ECs were transfected with reporter constructs bearing 3 tandem E-box1 or E-box2 sequences (see the Materials and Methods Section) cloned into the pGL-3 control vector. After transfection, cells were left untreated (control) or incubated with 10 μM quercetin. The relative luciferase activity of unstimulated HCAEC was set as 100%. Values were expressed as means ± SE of five individual experiments; $*P \leq 0.05$.

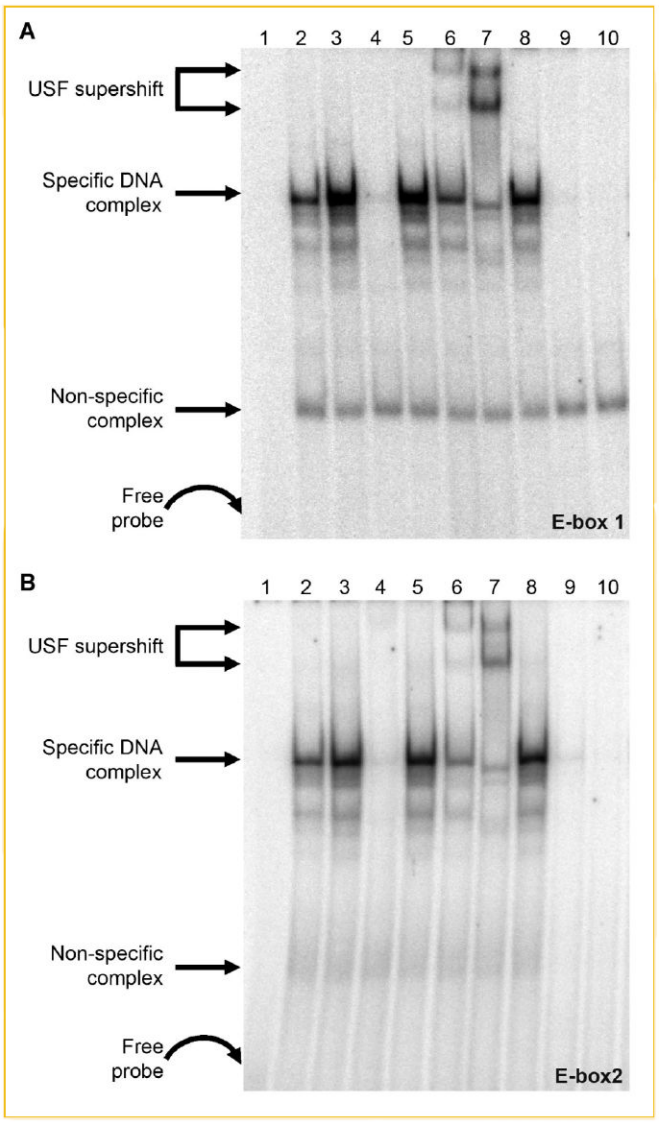

Fig. 5.

Identification of USF-1 and USF-2 as E-box-binding proteins. Nuclear proteins from untreated and Quercetin-treated cells were incubated with ^{32}P-labeled double-stranded oligonucleotides to E-box1 (A) or E-box2 (B). Lane 1, probe only; lane 2, untreated NE; lane 3, quercetin-treated NE; lane 4, quercetin-treated NE and 100-fold excess of unlabeled probe; lane 5, quercetin-treated NE and 100-fold excess of mutated E-box1 (A) or E-box2 (B); lane 6, quercetin-treated NE and USF-1 antibody; lane 7, quercetin-treated NE and USF-2 antibody; lane 8, quercetin-treated NE and c-Myc anibody; lane 9, unstimulated NE treated with lambda protein phosphatase; and lane 10, quercetin-stimulated NE treated with phosphatase. The two supershifted bands in (A) and (B) lane 7 and, to a lesser extent, lane 6 likely are due to the relative abundance of different complexes (i.e., USF-2 homodimers vs. USF-1/2 heterodimers) that bind to E-box1 and E-box2 as a function of stimulus conditions [e.g., Allen et al., 2005; Qi et al., 2006].

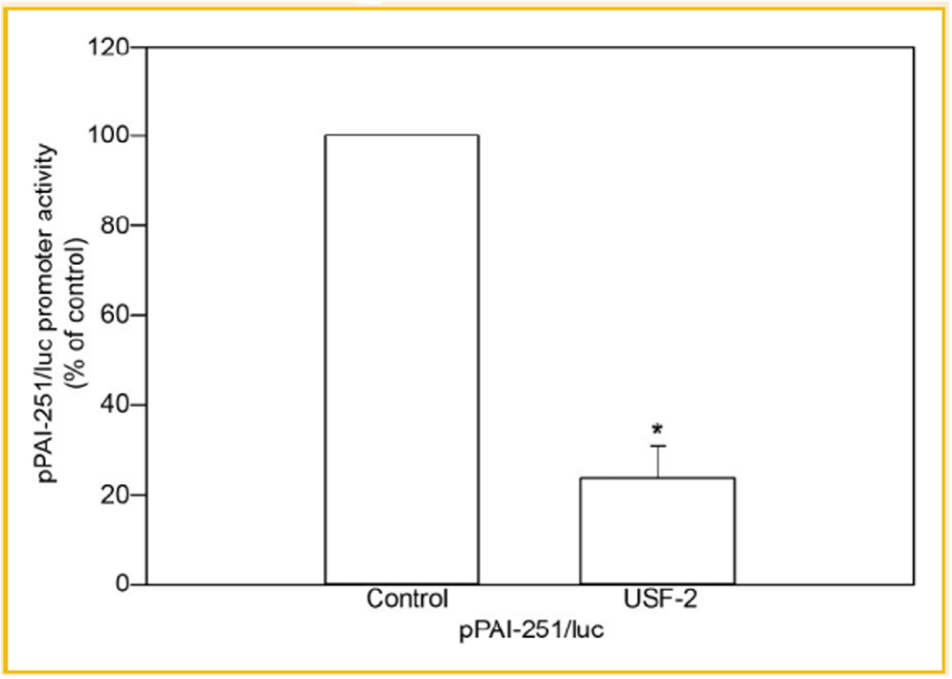

Fig. 6.

Effect of USF-2 on pPAI-251/luc transcription. pPAI-251/luc and pUSF-2/pcDNA were introduced simultaneously into HCAECs. Overexpression of USF-2 significantly decreased the relative luciferase activity of the pPAI-251/luc reporter. Signal from ECs transfected with pPAI-251 alone was set as 100%. Values were expressed as mean ± SE of five individual experiments; $*P \leq 30.05$.

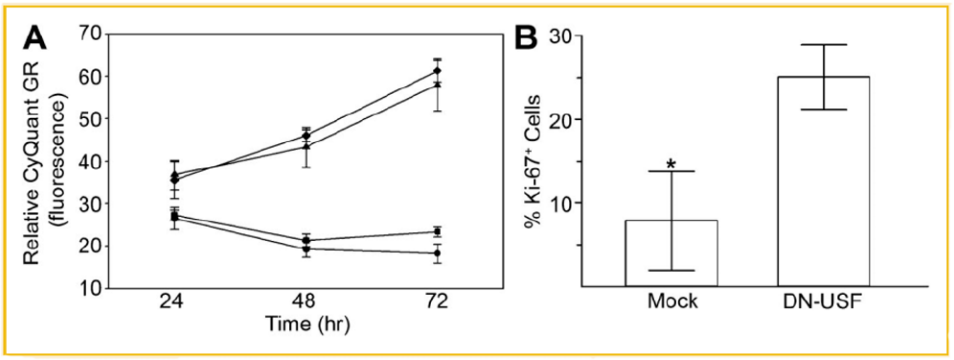

Fig. 7.

Effects of Quer and USF-2 on cell proliferation. A: ECs were incubated with Quer (10 μM) or transfected with the pUSF-2/pcDNA construct for 24, 48, and 72 h. Quercetin and overexpression of USF-2 significantly decreased proliferation. ◆, control media; ▼, pcDNA vector; ■, quercetin; ●, pUSF-2/pcDNA. Values were expressed as mean ± SE of five individual experiments; $*P \leq 0.05$. B: ECs were transfected with a DN-USF construct and the fraction of cells expressing Ki-67, a marker for cell proliferation, assessed immunocytochemically. Data plotted is the mean ± SE for three arbitrary-selected sets of 100 cells each demonstrating nuclear Ki-67 immunofluorescence. $*P \leq 0.05$. It was unlikely that apoptosis was a consideration in either the USF-2-dependent proliferative restriction or the growth response to DN-USF since DAPI staining (as a nuclear marker) did not indicate any significant change in the number of apoptotic cells.

TABLE I

E-box1 and E-box2 Probes Used in Electrophoretic Mobility Shift Assays

Oligo E-box1	5′-GTCTGGACACGTGGGGGAGTC-3′
Oligo mut E-box1	5′-GTCTGGACTTAATGGGGAGTC-3′
Oligo E-box2	5′-AGACAATCACGTGGCTGGCTG-3′
Oligo mut E-box2	5′-AGACAATCTTAATGCTGGCTG-3′

The wild-type E-box motifs are underlined and the mutant sequences underlined and highlighted in boldface. Only top strand illustrated.