

Ayumi Aurea Miyakawa, Maria de Lourdes Junqueira and José Eduardo Krieger

Physiol Genomics 17:107-113, 2004. First published Feb 10, 2004;

doi:10.1152/physiolgenomics.00169.2003

You might find this additional information useful...

This article cites 48 articles, 28 of which you can access free at:

<http://physiolgenomics.physiology.org/cgi/content/full/17/2/107#BIBL>

This article has been cited by 4 other HighWire hosted articles:

Laminar Shear Stress Regulates Endothelial Kinin B1 Receptor Expression and Function: Potential Implication in Atherogenesis

J. Duchene, C. Cayla, S. Vessillier, R. Scotland, K. Yamashiro, F. Lecomte, I. Syed, P. Vo, A. Marrelli, C. Pitzalis, F. Cipollone, J. Schanstra, J.-L. Bascands, A. J. Hobbs, M. Perretti and A. Ahluwalia

Arterioscler Thromb Vasc Biol, November 1, 2009; 29 (11): 1757-1763.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Induction of CRP3/MLP expression during vein arterialization is dependent on stretch rather than shear stress

L. C. G. Campos, A. A. Miyakawa, V. G. Barauna, L. Cardoso, T. F. Borin, L. A. d. O. Dallan and J. E. Krieger

Cardiovasc Res, July 1, 2009; 83 (1): 140-147.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

The role of the renin-angiotensin system and oxidative stress in spontaneously hypertensive rat mesenteric collateral growth impairment

S. J. Miller, L. E. Norton, M. P. Murphy, M. C. Dalsing and J. L. Unthank

Am J Physiol Heart Circ Physiol, May 1, 2007; 292 (5): H2523-H2531.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Endothelial KLF2 Links Local Arterial Shear Stress Levels to the Expression of Vascular Tone-Regulating Genes

R. J. Dekker, J. V. van Thienen, J. Rohlena, S. C. de Jager, Y. W. Elderkamp, J. Seppen, C. J.M. de Vries, E. A.L. Biessen, T. J.C. van Berkel, H. Pannekoek and A. J.G. Horrevoets

Am. J. Pathol., August 1, 2005; 167 (2): 609-618.

[\[Abstract\]](#) [\[Full Text\]](#) [\[PDF\]](#)

Updated information and services including high-resolution figures, can be found at:

<http://physiolgenomics.physiology.org/cgi/content/full/17/2/107>

Additional material and information about *Physiological Genomics* can be found at:

<http://www.the-aps.org/publications/pg>

This information is current as of November 23, 2009 .

Identification of two novel shear stress responsive elements in rat angiotensin I converting enzyme promoter

Ayumi Aurea Miyakawa, Maria de Lourdes Junqueira, and José Eduardo Krieger

Laboratory of Genetic and Molecular Cardiology, Heart Institute (InCor) and Internal Medicine

Department/LIM13, University of São Paulo Medical School, 05403-901 São Paulo SP, Brazil

Submitted 2 October 2003; accepted in final form 4 February 2004

Miyakawa, Ayumi Aurea, Maria de Lourdes Junqueira, and José Eduardo Krieger. Identification of two novel shear stress responsive elements in rat angiotensin I converting enzyme promoter. *Physiol Genomics* 17: 107–113, 2004. First published February 10, 2004; 10.1152/physiolgenomics.00169.2003.—Mechanical forces contribute to maintenance of cardiovascular homeostasis via the control of release and production of vasoactive substances. We demonstrated previously that shear stress decreases rat ACE activity and expression. Using a reporter gene approach and mutagenesis, we show now that the classic shear stress responsive element or SSRE (GAGACC) contained within 1,274 bp of this promoter is not functional in response to shear stress (15 dyn/cm², 18 h) [for the wild-type ACE promoter (WLuc), static control (C) = 107 ± 6.5%, shear stress (SS) = 65.9 ± 9.4%, *n* = 8; for the promoter with the classic SSRE mutated (WSS-mut), C = 100 ± 8.2%, SS = 60.2 ± 5.2%, *n* = 10, respectively]. Analysis of progressive deletion mutants unraveled a 57-bp fragment, position –251 to –195, from the transcription start site, containing functional SSRE (for WLuc, C = 107 ± 6.5%, SS = 65.9 ± 9.4%, *n* = 8; for 378, C = 100 ± 6.4%, SS = 60.4 ± 4.3%, *n* = 11; for 251, C = 99.7 ± 2.6%, SS = 63.2 ± 5.5%, *n* = 7; for 194, C = 104.6 ± 8.1%, SS = 92.4 ± 6.9%, *n* = 9). This fragment responded to shear stress even in the context of a heterologous promoter. Finally, functional analysis of mutated candidate regulatory elements identified by gel shift, DNase I footprint, and conservation of aligned sequences revealed that only the double mutant (Barbie/GAGA-mut) but not isolated disruption of the Barbie (WBarbie-mut) or the GAGA (WGAGA-mut) prevented the shear-stress-induced response (for Barbie/GAGA-mut, C = 97.9 ± 5%, SS = 99.4 ± 7.2%, *n* = 6; for WBarbie-mut, C = 106.1 ± 8.6%, SS = 65.9 ± 9.4%, *n* = 6; for WGAGA-mut, C = 100.1 ± 2.9%, SS = 66.7 ± 1.6, *n* = 6). Taken together, these data provide direct evidence for the new role of Barbie and GAGA boxes in mediating the shear-stress-induced downregulation of rat ACE expression and demonstrate that the classic SSRE (GAGACC) is not functional under the experimental conditions tested.

laminar shear stress; promoter analysis; endothelial cell

THE INTERPLAY BETWEEN the hemodynamic environment and the endothelium plays a critical role in vascular function. The endothelial cells separating the blood and the vascular wall are subjected to shear force that can induce both short-term vasomotor and long-term modifications in vessel structure in response to blood flow changes (4). In vivo observations show that atherosclerotic lesions typically appear in bifurcation points and areas of curvature where flow pattern is disrupted from laminar to turbulent decreasing shear stress (10). Inter-

estingly, the atherosclerotic lesions develop preferentially in the vascular segment subjected to lower shear, raising the possibility that shear stress is a contributing factor for the development/maintenance of the complex processes underlying atherosclerosis (21). Evidence for the direct action of shear stress on endothelial function comes primarily from in vitro studies, where it has been demonstrated that genes important to vascular endothelial physiology such as nitric oxide synthase (NOS) (49), endothelin-1 (22), fibroblast growth factor (FGF) (33), platelet-derived growth factor (PDGF) (14), thrombomodulin (31) are all influenced by this stimulus. However, the mechanism whereby endothelial cells sense and transduce the shear stress stimulus remains unclear. Several candidate shear stress sensors in endothelial cells have been postulated, which include integrins (15), ion channels (37, 48), G proteins (41, 35), and MAPK (16, 17) coupled receptors. The first proposed shear stress responsive element (SSRE) was identified in the PDGF gene and consists of a 6-bp core element, GAGACC, that can interact with NF-κB transcription factor (42, 20), but other transcription factors have also been described to be activated by shear such as AP-1 (23), Sp-1 (29), and Erg-1 (19).

Previously, we have demonstrated that shear stress suppresses angiotensin I converting enzyme (ACE) expression and enzyme activity in vivo and in vitro. In addition, using a reporter gene approach, we provided evidence that a fragment of 1,274 bp upstream of the transcription initiation site of the rat ACE promoter contained SSRE sequences (43). This response is not mediated by NO, although NO can influence ACE basal levels under static condition (39). The 1,274-bp rat ACE promoter under study contains the classic SSRE, and it was the first candidate tested. Surprisingly, we demonstrated that the classic SSRE is not functional in rat ACE promoter under the experimental conditions tested. In contrast, we describe that shear-stress-induced ACE downregulation involves two *cis*-acting elements, Barbie and GAGA boxes, which have not been associated to shear-stress-induced responses until now.

MATERIAL AND METHODS

Cell culture. Rabbit aortic endothelial cells (REC) (3) were grown in culture using F-12 Coon's medium (Sigma Chemical, St. Louis, MO) containing 10% FBS (GIBCO; Invitrogen, Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO, Invitrogen). These cells were passaged by exposure to pancreatin for 30 min and maintain characteristics of endothelial cells such as morphological appearance, expression of ACE and factor VIII, and capacity to internalize acetylated LDL (acLDL) (data not shown).

ACE promoter constructs and mutants. Luciferase reporter plasmid constructs were made by subcloning the rat ACE promoter fragments and mutants into the backbone of pGL2 Basic vector (Promega,

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

Address for reprint requests and other correspondence: J. E. Krieger, Laboratory of Genetic and Molecular Cardiology, Heart Institute (InCor), São Paulo Univ. Medical School, Av. Dr. Enéas C. Aguiar, 44-10 andar, 05403-901 São Paulo SP, Brazil (E-mail: krieger@incor.usp.br).

Table 1. Primers sequences used to mutate putative sites in rat ACE promoter

Name	Sequence
WSS-mut	F: 5' TGTGGATATCTGAGGTGACTTG 3' R: 5' CAAGTCACCTCAGATATCCACA 3'
WBarbie-mut	F: 5' GCAGCCCGCAGGGATCCGGATCCCTTCCTCAGC 3' R: 5' GCTGAGGAAGGGATCCGGATCCCTGCGGGCTGC 3'
WGAGA-mut	F: 5' CTAGCTTCCTCTGCAGCTGCAGACCTAACCTCATC 3' R: 5' GATGAGTTAGGTCTGCAGCTGCAGGAAGCTAG 3'

The underlined sequences represent the mutation performed with insertion of enzyme restriction sites: *EcoRV* in WSS-mut, *BamHI* in WBarbie-mut, and *PstI* in WGAGA-mut. F and R are forward and reverse primers, respectively. WSS-mut, promoter with the classic SSRE mutated; WBarbie-mut, promoter with the Barbie box consensus mutated; and WGAGA-mut, promoter with the GAGA box consensus mutated.

Madison, WI). The 1,274-bp ACE promoter fragment was cloned in the *MluI* site, and *BstXI*, *HaeIII*, and *SacI* digestions were used to generate 5' deletion mutants containing 378-, 251-, and 194-bp fragments, respectively. The site-directed mutageneses were performed in the 1,274-bp fragment by the method of PCR overlapping primers (34). The primers sequences used to generate the mutations in consensus sequences are shown in Table 1. Positive control for the study of the classic SSRE present in the rat ACE promoter (SSRE-Luc) was made by the construction of hybrid of two tandem repeats of the sequence GAGACC upstream from SV40 promoter into the pGL2 promoter backbone vector (Promega). All mutants were certified by enzymatic sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA).

Stable transfection with ACE promoter constructs. REC were stably transfected by the calcium phosphate method (1) with the constructs obtained as described above. Cells were cotransfected with pSV7Neo, a neomycin-resistant plasmid, and selected with G418 (GIBCO, Invitrogen), from which cell clones were selected on the basis of luciferase production and maintenance of cell appearance. For each construct at least two clones were examined.

Shear stress protocols. All cells were submitted to controlled shear using a cone plate viscometer kindly provided by G. H. Gibbons (32, 33). Cells (3×10^6) were plated in 150-mm dishes with 20 ml for 48 h, then the medium was changed and the cells were submitted to shear at 15 dyn/cm² for 18 h. At the end of the experiment the cells were washed with PBS (in mmol/l: 137 NaCl, 2.7 KCl, 4.3 Na₂HPO₄, and 1.4 KH₂PO₄) and lysed in 2 ml of borate buffer (in mol/l: 0.5 boric acid, 1.125 NaCl, and 0.1% Triton X-100, pH 8.3). The samples were stored at -80°C until ACE and luciferase activities were determined as described below.

NO production, assessed as the amount of nitrite and nitrate in the media, was used as a positive control for shear-induced response (e.g., 4-fold increase in nitrite and nitrate content in the media comparing 15 dyn/cm², 18 h vs. static conditions).

Luciferase activity. Luciferase activity was measured using a commercially available kit (Promega Luciferase Assay System). Briefly, 5 µl of lysed cells were added to the luciferin substrate and immediately assayed using a luminometer (Monolight 2010, Analytical luminance laboratory). The results were normalized by the amount of DNA present in the sample using the dye Hoechst 33258 (24).

In all promoter function studies the expected endogenous rabbit ACE gene downregulation induced by shear stress was assayed, and only samples where ACE activity (38) decreased by at least 10% were further analyzed for luciferase activity.

Gel mobility shift assay. Binding of nuclear extracts to DNA was assessed as described by Ausubel et al. (1). Briefly, 57-bp fragments, wild type and mutated, were end-labeled with ³²P and incubated with 10 µg of sheared and non-sheared nuclear proteins, in the presence of 0.1 µg/µl of poly-dIdC, 10 mM NaPO₄; 50% glycerol; 2.5 mM EDTA, 5 mM MgCl₂.

DNase I footprinting analysis. DNase I footprinting analyses were performed as described by Ausubel et al. (1). The wild-type 57-bp fragment was end-labeled with ³²P and purified using 5% polyacrylamide gel electrophoresis. The binding reaction was performed with 60,000 cpm of labeled DNA and 5–20 µg of nuclear proteins. After incubation with 1 U of DNase I, the hydrolyzed products were analyzed in 8% polyacrylamide gel. Chemical sequencing reaction (G+A) was carried out by standard procedure.

Sequence alignment and candidate transcription factor search. Rat (AY344961), mouse (M34433), and human (M34434) ACE promoters were aligned with CLUSTAL W at <http://transfac.gbf.de/>. Detection of transcription factor binding sites was performed with MatInspector V2.2 public domain based on TRANSFAC 4.0 (46) (<http://transfac.gbf.de/TRANSFAC/>).

Statistical methods. Data from multiple experiments are expressed as means ± SE or as percentage change. For each functional experiment at least two cell clones were used and repeated twice. The results were analyzed by two-tailed *t*-test (static vs. shear), and values of *P* < 0.05 were considered significant.

RESULTS

Analysis of classic SSRE in rat ACE promoter. The classic GAGACC sequence located at position -793/-788 was the first SSRE candidate examined in the rat ACE promoter. To confirm the functionality of the core SSRE, the consensus sequence GAGACC was mutated to GATATC and the promoter activity was analyzed (WSS-mut, Fig. 1A). As expected, the activity of wild-type promoter, WLuc, decreased 34% when subjected to shear stress of 15 dyn/cm² for 18 h compared with static control (C = 107 ± 6.5%, SS = 65.9 ± 9.4%, *n* = 8). Surprisingly, WSS-mut activity was also suppressed by 40% in sheared condition (C = 100 ± 8.2%, SS = 60.2 ±

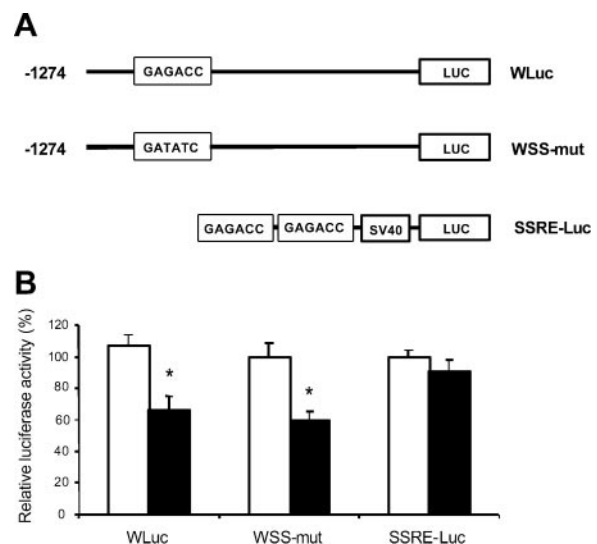


Fig. 1. Classic shear stress responsive element (SSRE) in rat angiotensin I converting enzyme (ACE) promoter function. A: constructs used to transfect rabbit aortic endothelial cells (REC) cells. WLuc is the wild-type ACE promoter, WSS-mut is the promoter with the classic SSRE mutated, and SSRE-Luc is the positive control bearing two tandem repeats of the classic SSRE upstream of the SV40 promoter into the pGL2 promoter backbone vector. B: luciferase activity of REC cells transfected with constructs showed in A. The experiments were performed in at least 2 different cell clones for each construct, and each bar represents mean ± SE of 8–10 experiments. Open bars refer to static control cells, and solid bars refer to cells submitted to shear stress of 15 dyn/cm² for 18 h; the results are normalized by the static values. **P* < 0.05 in relation to static control.

5.2%, $n = 10$), showing the same response as the wild-type WLuc promoter. In addition, the positive control SSRE-Luc, which contains two tandem repeats of the classic SSRE upstream of the SV40 promoter in the pGL2 promoter backbone vector, did not respond to the shear stress stimulation ($C = 100 \pm 4.1\%$, $SS = 90.9 \pm 6.9\%$, $n = 9$) (Fig. 1B). These results indicate that the classic SSRE present in the rat ACE promoter is not functional under the experimental conditions tested.

Progressive deletion of rat ACE promoter. To identify the functional SSRE in the 1,274 bp of ACE promoter gene, progressive deletion mutants were made obtaining fragments of 378, 251, and 194 bp, which were stably transfected into clones of REC for further analysis (Fig. 2A). Shear stress stimulus of 15 dyn/cm² for 18 h decreased luciferase activity by 40% and 37% compared with static condition in the fragments 378 and 251, respectively. These values are of same magnitude obtained with the full fragment 1274. In contrast, the responsiveness to shear stress was abolished in the fragment 194 indicating that functional SSRE sequences are contained within the 57-bp interval, position -251 to -195 (for WLuc, $C = 107 \pm 6.5\%$, $SS = 65.9 \pm 9.4\%$, $n = 8$; for 378, $C = 100 \pm 6.4\%$, $SS = 60.4 \pm 4.3\%$, $n = 11$; for 251, $C = 99.7 \pm 2.6\%$, $SS = 63.2 \pm 5.5\%$, $n = 7$; for 194, $C = 104.6 \pm 8.1\%$, $SS = 92.4 \pm 6.9\%$, $n = 9$) (Fig. 2B). These findings were further tested by subcloning the 57-bp fragment upstream of a heterologous SV40 promoter (57Luc) in the backbone of pGL2 promoter vector. When this construct was subjected to shear stress, luciferase activity decreased by 46% in relation to static control (Fig. 2C), which is consistent with the idea that the 57-bp fragment indeed contains SSRE sequences ($C = 100 \pm 7.9\%$, $SS = 54.5 \pm 6.6\%$, $n = 5$).

Protein interaction in the 57-bp fragment. The binding of nuclear proteins extracted from static and sheared REC to the 57-bp fragment was verified by gel mobility shift assay and revealed at least three band shifts (Fig. 3A). Interestingly, the interaction pattern remained the same when using static or sheared nuclear proteins, suggesting that there is no induction

of transcription factor or that additional proteins of low abundance or low affinity for the DNA under current experimental conditions are involved. Alternatively, one may speculate that under the experimental condition tested shear-stress-induced response is associated with modification of already-bound proteins. DNase I footprinting analysis was performed to determine transcription factor interaction to the 57-bp fragment using nuclear extracts of static REC, since sheared and non-sheared cells presented the same pattern of interaction. One DNA region was protected from DNase I digestion, located within the interval -218/-215, which corresponds to the sequence CTTT (Fig. 3B). This sequence is known as Barbie box, because of its interaction with barbiturate-inducible regulatory factors (11). Again, additional proteins with low abundance or low affinity would not be identified under the current experimental conditions. Based on this observation, a competition gel shift assay was performed using the 57-bp fragment mutated in Barbie box (57Barbie-mut) and 2 tandem repeats of Barbie box (BARBIE) as competitors. The 57Barbie-mut did not inhibit the complex formation by nuclear proteins and the 57-bp fragment, whereas the BARBIE competitor abolished this interaction (Fig. 3C). Taken together, these data give support to the DNase I footprinting finding, indicating the involvement of Barbie box.

Functional analysis of BARBIE box. To evaluate the functional role of the Barbie box, the core sequence of Barbie and adjacent sequences were mutated in the rat ACE promoter (WBarbie-mut, Fig. 4A). Unexpectedly, this mutation alone did not affect the response of ACE promoter since shear stress stimulus in WBarbie-mut still led to a significant 40% reduction in activity ($C = 106.1 \pm 8.6\%$, $SS = 65.9 \pm 9.4\%$, $n = 6$), which is comparable to the response of the wild-type promoter (Fig. 4B).

Study of putative binding sites in the 57-bp fragment. The gel shift mobility assay suggested more than one protein interaction site in the 57-bp fragment, although the DNase I footprint revealed only the Barbie box interaction (Fig. 3). Since Barbie-mut still responded to shear stress and functional

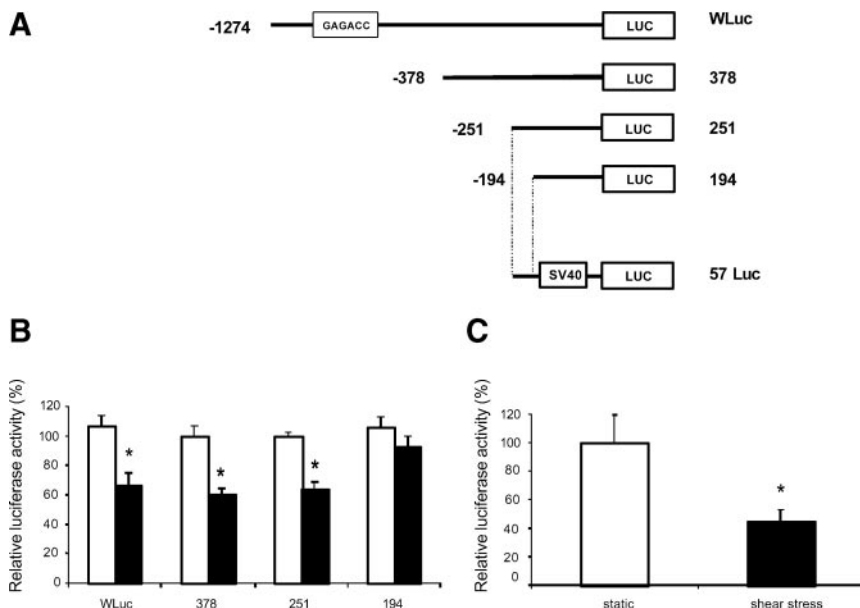


Fig. 2. Shear-stress-induced responsiveness in rat ACE promoter deletion mutants. A: series of deletion mutants of the rat ACE promoter coupled to luciferase gene used to transfect REC cells. 57Luc is the 57-bp fragment, -251 and -195 interval, cloned upstream from the SV40 promoter in the pGL2 promoter backbone vector. B and C: luciferase activity of REC cells transfected with deletion mutations (B) and 57Luc construct (C). The experiments were performed in at least 2 different cell clones for each construct and each bar represents mean \pm SE of 7–10 experiments. Open bars refer to static control cells, and solid bars refer to cells submitted to shear stress of 15 dyn/cm² for 18 h, the results are normalized by the static values. * $P < 0.05$ in relation to static control.

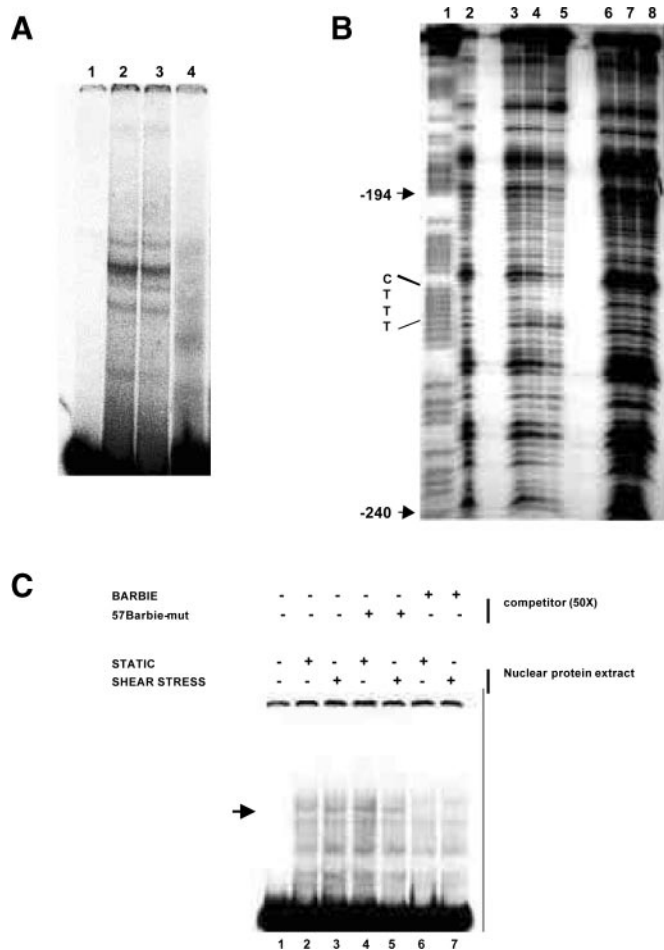


Fig. 3. Protein interaction with the 57-bp fragment from rat ACE promoter. **A:** gel shift mobility assay using the 57-bp fragment as probe. *Lane 1:* no nuclear extract. *Lane 2:* 10 μ g of nuclear extract from static REC. *Lane 3:* 10 μ g of nuclear extract from sheared REC (15 dyn/cm², 18 h). *Lane 4:* competition with 50 \times of cold 57 fragment. **B:** DNase I footprinting. The 57-bp fragment was end-labeled with ³²P, incubated with nuclear protein extract of REC or BSA, and then subjected to DNase I digestion. *Lane 1:* chemical sequencing reaction (G+A). *Lane 2:* no nuclear extract. *Lanes 3–5:* 5, 10, and 20 μ g of REC nuclear extract, respectively. *Lanes 6–8:* 5, 10, and 20 μ g of BSA, respectively. **C:** gel shift mobility assay. The 57-bp fragment was end-labeled with ³²P, incubated with nuclear protein from sheared and non-sheared endothelial cells in the presence or absence of cold competitor: 57-bp fragment mutated in Barbie box (57Barbie-mut) or 2 tandem repeats of ACE Barbie box consensus (BARBIE).

experiments with 57Luc showed regulatory sequences within this fragment, we performed an analysis of consensus sequences within this promoter fragment to identify other potential regulatory sequences that could be influencing the shear stress response. Three additional putative sites were revealed: AP-4, AP-2, and GAGA box (Fig. 5A). Alignment of rat, mouse, and human ACE promoters showed that among these putative sites present in the 57-bp fragment, Barbie and GAGA boxes are the only ones conserved across the different species (Fig. 5B). In addition, potential Barbie and GAGA boxes are also found in the rabbit promoter between $-96/-76$ (50 bp from TATA box), which is closer to the TATA box than in the rat promoter, where these are between $-220/-196$ (174 bp from TATA box) (data not shown).

Study of Barbie and GAGA box functionality in ACE promoter. Based on promoter alignment and consensus sequences conservation, GAGA box was mutated in the rat ACE promoter (WGAGA-mut), and also a double mutant was produced including Barbie and GAGA boxes (Barbie/GAGA-mut) (Fig. 6A). Similar to what was observed with isolated mutation on Barbie box (Fig. 4), the WGAGA-mut construct decreased luciferase activity by 33% in response to shear stress ($C = 100.1 \pm 2.9\%$, $SS = 66.7 \pm 1.6$, $n = 6$). On the other hand, the shear-stress-induced response failed to appear with the double mutant (for Barbie/GAGA-mut, $C = 97.9 \pm 5\%$, $SS = 99.4 \pm 7.2\%$, $n = 6$), suggesting that Barbie and GAGA boxes are necessary for the abrogation of shear-stress-induced response (Fig. 6B).

DISCUSSION

Previously, we have demonstrated that shear stress suppresses ACE activity and expression in vitro and in vivo. Furthermore, we used endothelial cells stably transfected with 1,274 bp of rat ACE promoter controlling the luciferase reporter gene (WLuc) to provide evidence that sequences contained within this fragment influenced, at least in part, the shear-stress-induced response (43).

In this study, we demonstrated that the classic SSRE (GAGACC) present in rat ACE promoter ($-793/-788$) is not functional. Mutation and deletion of this consensus GAGACC sequence, WSS-mut, and the 378 construct, respectively, did not affect the responsiveness of ACE promoter to shear stress (Figs. 1 and 2). These data provide strong evidence against the role of this consensus sequence (GAGACC) on the shear stress regulation of the rat ACE promoter under the experimental condition tested. It is noteworthy that genes regulated by shear stress, such as TGF β 1, have the classic SSRE in their promoter, but their response to shear stress does not require this sequence (36). Indeed it suggests that regulatory sequences other than GAGACC may be involved in shear-induced re-

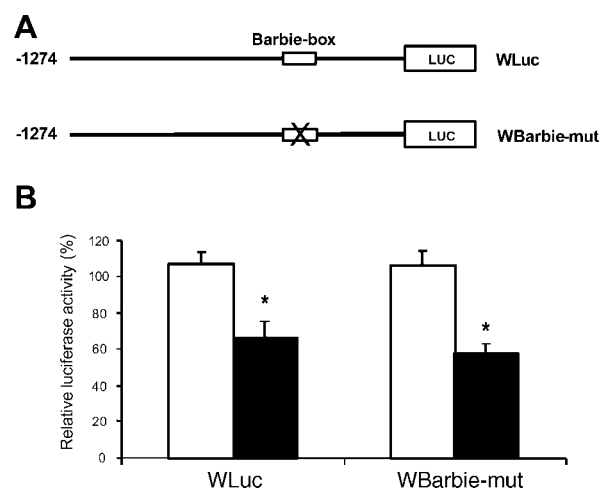


Fig. 4. Functional study of Barbie box in rat ACE promoter. **A:** constructs used to transfect REC cells. WLuc is the wild-type rat ACE promoter; WBarbie-mut is the construct with the Barbie box consensus mutated. **B:** luciferase activity of REC cells transfected with WBarbie-mut. The experiments were performed in at least 2 different cell clones for each construct, and each bar represents mean \pm SE of 6 experiments. Open bars refer to static control cells, and solid bars refer to cells submitted to shear stress of 15 dyn/cm² for 18 h; the results are normalized by the static values. * $P < 0.05$ in relation to static control.

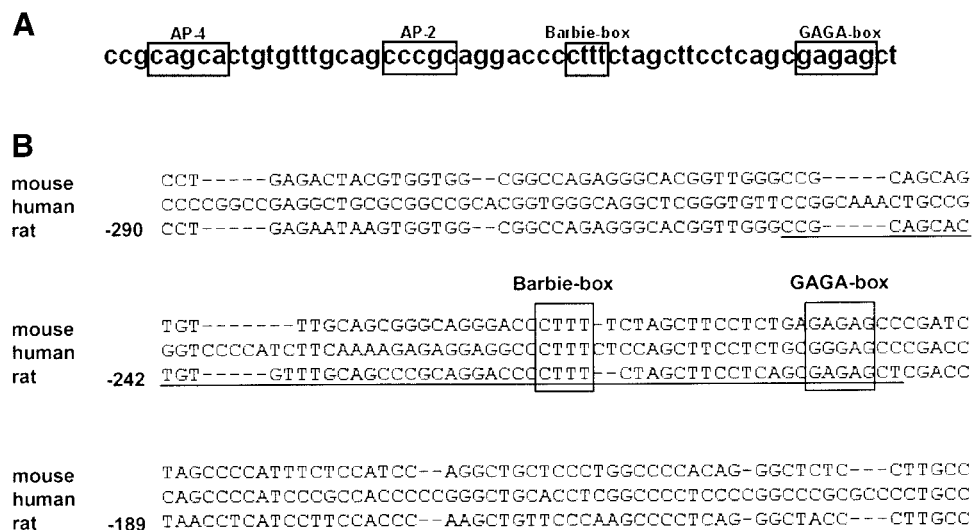


Fig. 5. A: putative sites for transcription factors present in the 57-bp fragment. B: human, mouse, and rat ACE promoters aligned. The underlined bases are the 57-bp fragment, and the boxes show the conservation of Barbie and GAGA box.

sponse. Further characterization of promoter function by progressive deletion analysis demonstrated the presence of SSRE sequences within a fragment of 57 bp (Fig. 2). Using combinations of DNase I footprint, gel shift mobility assays and mutations experiments, two novel *cis*-elements were identified to play a role in shear-stress-induced ACE downregulation, namely, Barbie and GAGA boxes.

Barbie box element is present in eukaryotic and prokaryotic genes regulated by barbiturate, such as cytochrome *P*-450 genes of *Bacillus megaterium* and rat (11). In *B. megaterium*

the Bm3R1 is the protein that binds to Barbie box and represses gene transcription in cytochrome *P*-450 gene. This binding is modulated by two other factors, Bm1P1 and/or Bm1P2, that interact directly with Bm3R1 and alter its binding properties to Barbie box or can compete with Bm3R1 to the binding site (27, 28). In rats, a model for transcriptional regulation of the CYP2B1/B2 was suggested by Prabhu et al. (40). Under non-induced condition the transcription factor is predominantly in the dephosphorylated state, permitting only basal transcription. Phenobarbital treatment leads to phosphorylation of the factor shifting the equilibrium toward binding to the Barbie box, leading to activation of transcription. It is interesting to note that in bacteria the Barbie box element has negative regulatory function and in rat positive.

GAGA box is a region rich in G and A, it was first described in *Drosophila* heat shock protein and in histone gene promoters (9). A protein called GAGA factor that was first implicated in chromatin structure and remodeling binds to the GAGA box (8). Now it has been suggested that the GAGA factor promotes transcription by blocking the repressive effects of histones, then facilitating transcription factor IID (TFIID) binding and the recruitment of RNA polymerase II, forming the transcription initiation complex (TIC) (25). Alternatively, it provides a protein bridge, permitting interaction with other binding sequences of the promoter and modulating transcription (18, 30). In addition, GAGA factor may be more like a transcriptional potentiator/coactivator of Sp1 than a transcription factor itself (2). The involvement of GAGA box in vertebrate genes such as serine protease inhibitor-2.1 (26), angiotensin II AT₁ receptor (47), and vasopressin V_{1b} receptor (44) has been reported recently. Mammalian GAGA factor has not yet been isolated; however, the *Drosophila* GAGA factor is capable to stimulate rat V_{1b} vasopressin receptor promoter activity (44).

In the present work, we were able to provide evidence that the integrity of both Barbie and GAGA boxes has to be disturbed to abrogate the shear-stress-induced ACE gene downregulation (Fig. 6). The shear stress mechanotransduction is not yet fully elucidated and, for the first time, we provide evidence for two novel players in this process with regard to rat ACE gene expression control. Interestingly, recent findings have shown that shear stress induces CYP1B1 (6) and hsp60

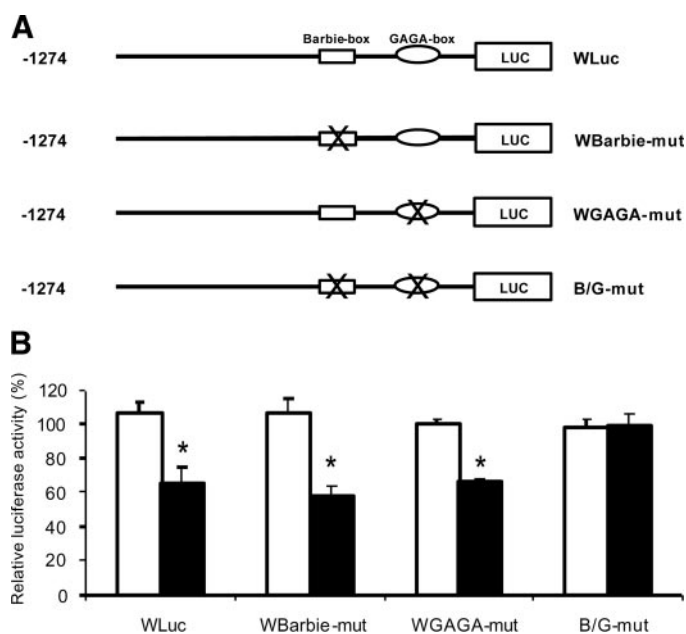


Fig. 6. Functional analyses of Barbie box and GAGA box in rat ACE promoter. A: constructs used to transfect REC cells. WLuc is the wild-type ACE promoter, WBarbie-mut is the promoter with the Barbie box consensus mutated, and WGAGA-mut is the promoter with the GAGA box consensus mutated. B: luciferase activity of REC cells transfected with constructs showed in A. The experiments were performed in at least 2 different cell clones for each construct, and each bar represents mean \pm SE of 6 experiments. Open bars refer to static control cells, and solid bars refer to cells submitted to shear stress of 15 dyn/cm² for 18 h; the results are normalized by the static values. **P* < 0.05 in relation to static control.

(12) expression in human umbilical vein endothelial cells. The Barbie and GAGA boxes, respectively, have functional importance in regulating CYP1B1 and hsp60 expression as discussed above, and the importance of these elements under shear stimulus must be investigated. The endothelial response to shear stress is complex and involves modulation of several genes. It has been demonstrated that shear stress regulates vasoactive substances, growth factors, adhesion molecules, cytoskeleton molecules, transcription factors and others (4). It is reasonable to consider that shear stress participates in the maintenance of endothelial homeostasis and may play important role in pathological vascular events. Atherosclerosis has been implicated with disruption in flow patterns in the vascular tree since the lesions preferentially appear in regions of low shear stress such as bifurcations and curvatures (21). Techniques that allow the simultaneous analysis of hundreds of known or unknown genes may be critical to identify the variety of players involved in this complex response (45). These approaches may contribute to elucidate the mechanisms underlying shear-stress-induced responses and how flow-responsive genes act coordinately in physiopathological events.

Increased ACE within the atherosclerotic plaque is believed to be important for initiation and/or progression of the lesion (7) which can be ameliorated by the use of the ACE inhibitors or angiotensin AT₁ receptor antagonist (5, 13). The specific contribution of inflammatory cells or physical factors, such as shear stress, in initiation or sustaining this process, remains elusive, but understanding the molecular mechanism underlining ACE gene regulation under this condition may provide new therapeutic opportunities. Taken together, these results provide evidence for the new role of Barbie box and GAGA box in mediating the shear-stress-induced downregulation of rat ACE promoter function and demonstrate that the classic SSRE (GAGACC) is not functional in the rat ACE promoter under the experimental conditions tested.

GRANTS

A. A. Miyakawa is recipient of fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 98/11645-0 and 00/09485-7). This work was funded by grants from FAPESP (01/00009-0) and from the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq, 471219/01-0)

REFERENCES

- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. *Current Protocols in Molecular Biology*. Hoboken, NJ: Wiley, 1990, vol. 1, unit 9.1.
- Bevilacqua A, Fiorenza MT, and Mangia F. A developmentally regulated GAGA box-binding factor and Sp1 are required for transcription of the hsp70.1 gene at the onset of mouse zygotic genome activation. *Development* 127: 1541–1551, 2000.
- Buonassisi V and Venter JC. Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc Natl Acad Sci USA* 73: 1612–1616, 1976.
- Davies PF. Flow-mediated endothelial mechanotransduction. *Physiol Rev* 75: 519–560, 1995.
- de las Heras N, Aragoncillo P, Maeso R, Vazquez-Perez S, Navarro-Cid J, DeGasparo M, Mann J, Ruilope LM, Cachofeiro V, and Lahera V. AT(1) receptor antagonism reduces endothelial dysfunction and intimal thickening in atherosclerotic rabbits. *Hypertension* 34: 969–975, 1999.
- Dekker RJ, van Soest S, Fontijn RD, Salamanca S, de Groot PG, VanBavel E, Pannekoek H, and Horrevoets AJ. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLF2). *Blood* 100: 1689–1698, 2002.
- Diet F, Pratt RE, Berry GJ, Momose N, Gibbons GH, and Dzau VJ. Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation* 94: 2756–2767, 1996.
- Farkas G, Gausz J, Galloni M, Reuter G, Gyurkovics H, and Karch F. The Trithorax-like gene encodes the *Drosophila* GAGA factor. *Nature* 371: 806–808, 1994.
- Gilmour DS, Thomas GH, and Elgin SC. *Drosophila* nuclear proteins bind to regions of alternating C and T residues in gene promoters. *Science* 245: 1487–1490, 1989.
- Glagov S, Zarins C, Giddens DP, and Ku DN. Hemodynamics and atherosclerosis. *Arch Pathol Lab Med* 112: 1018–1031, 1988.
- He JS and Fulco AJ. A barbiturate-regulated protein binding to a common sequence in the cytochrome P450 genes of rodents and bacteria. *J Biol Chem* 266: 7864–7869, 1991.
- Hochleitner BW, Hochleitner EO, Obrist P, Eberl T, Amberger A, Xu Q, Margreiter R, and Wick G. Fluid shear stress induces heat shock protein 60 expression in endothelial cells in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 20: 617–623, 2000.
- Hope S, Brecher P, and Chobanian AV. Comparison of the effects of AT1 receptor blockade and angiotensin converting enzyme inhibition on atherosclerosis. *Am J Hypertens* 12: 28–34, 1999.
- Hsieh HJ, Li NQ, and Frangos JA. Shear-induced platelet-derived growth factor gene expression in human endothelial cell is mediated by protein kinase C. *J Cell Physiol* 150: 552–558, 1992.
- Ingber DE. Tensigrity: the architectural basis of cellular mechanotransduction. *Annu Rev Physiol* 59: 575–599, 1997.
- Jalali S, Li YS, Sotoudeh M, Yuan S, Li S, Chien S, and Shyy JYJ. Shear stress activates p60src-Ras-MAPK signaling pathway in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 18: 227–234, 1998.
- Jo H, Sipos K, Go YM, Law R, Rong J, and McDonald JM. Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. *J Biol Chem* 272: 1395–1401, 1997.
- Katsani KR, Hajibagheri MA, and Verrijzer CP. Co-operative DNA binding by GAGA transcription factor requires the conserved BTB/POZ domain and reorganizes promoter topology. *EMBO J* 18: 698–708, 1999.
- Khachigian LM, Anderson KR, Halnon NJ, Gimbrone MA Jr, Resnick N, and Collins T. Erg-1 is activated in endothelial cells exposed to fluid shear stress and interacts with a novel shear stress response element in the PDGF A-chain promoter. *Arterioscler Thromb Vasc Biol* 17: 2280–2286, 1997.
- Khachigian LM, Resnick N, Gimbrone MA Jr, and Collins T. Nuclear factor- κ B interacts functionally with the platelet-derived growth factor B-chain shear stress response element in vascular endothelial cells exposed to fluid shear stress. *J Clin Invest* 96: 1169–1175, 1995.
- Ku DN, Giddens DP, Zarins CK, and Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis* 5: 293–302, 1985.
- Kuchan MJ and Frangos JA. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. *Am J Physiol Heart Circ Physiol* 264: H150–H156, 1993.
- Lan Q, Mercurius KO, and Davies PF. Stimulation of transcription factors NF κ B and AP1 in endothelial cells subjected to shear stress. *Biochem Biophys Res Commun* 201: 950–956, 1994.
- Latt AS and Stetten G. Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J Histochem Cytochem* 24: 24–33, 1976.
- Leibovitch BA, Lu Q, Benjamin LR, Liu Y, Gilmour DS, and Elgin SC. GAGA factor and the TFIID complex collaborate in generating an open chromatin structure at the *Drosophila melanogaster* hsp26 promoter. *Mol Cell Biol* 22: 6148–6157, 2002.
- Leverrier S, Cinato E, Paul C, Derancourt J, Bemark M, Leanderson T, and Legraverend C. Purification and cloning of type A/B hnRNP proteins involved in transcriptional activation from the Rat spi 2 gene GAGA box. *Biol Chem* 381: 1031–1040, 2000.
- Liang Q and Fulco AJ. Transcriptional regulation of the genes encoding cytochromes P450BM-1 and P450BM-3 in *Bacillus megaterium* by the binding of Bm3R1 repressor to Barbie box elements operator sites. *J Biol Chem* 270: 18606–18614, 1995.
- Liang Q, He JS, and Fulco AJ. The role of Barbie box sequences as cis-acting elements involved in the barbiturate-mediated induction of cytochromes P450BM-1 and P450BM-3 in *Bacillus megaterium*. *J Biol Chem* 270: 4438–4450, 1995.
- Lin MC, Almus-Jacobs F, Chen HH, Parry GCN, Mackman N, and Shyy JYJ. Shear stress induction of the tissue factor gene. *J Clin Invest* 99: 737–744, 1997.

30. **Mahmoudi T, Katsani KR, and Verrijzer CP.** GAGA can mediate enhancer function in trans by linking two separate DNA molecules. *EMBO J* 21: 1775–1781, 2002.
31. **Malek AM, Jackman R, Rosenberg RD, and Izumo S.** Endothelial expression of thrombomodulin is reversibly regulated by fluid shear stress. *Circ Res* 74: 852–860, 1994.
32. **Malek A and Izumo S.** Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *Am J Physiol Cell Physiol* 263: C389–C396, 1992.
33. **Malek AM, Gibbons GH, Dzau VJ, and Izumo S.** Fluid shear stress differentially modulates expression of genes encoding basic fibroblast growth factor and platelet-derived growth factor B chain in vascular endothelium. *J Clin Invest* 92: 2013–2021, 1993.
34. **Motum PI, Lindeman R, Harvey MP, and Trent RJ.** Comparative studies of nondeletional HPFH gamma-globin gene promoters. *Exp Hematol* 21: 852–858, 1993.
35. **Nollert MU, Eskin SG, and McIntire LV.** Shear stress increases inositol trisphosphate levels in human endothelial cells. *Biochem Biophys Res Commun* 170: 281–289, 1990.
36. **Ohno M, Cooke JP, Dzau VJ, and Gibbons GH.** Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production. Modulation by potassium channel blockade. *J Clin Invest* 95: 1363–1369, 1995.
37. **Olensen SP, Clapham DE, and Davis PF.** Haemodynamics shear stress activates K^+ current in vascular endothelial cells. *Nature* 331: 168–170, 1988.
38. **Oliveira EM, Santos RAS, and Krieger JE.** Standardization of a fluorimetric assay for the determination of tissue angiotensin-converting enzyme activity in rats. *Braz J Med Biol Res* 33: 755–764, 2000.
39. **Pertrini CM, Miyakawa AA, Laurindo FRM, and Krieger JE.** Nitric oxide regulates angiotensin-I converting enzyme under static condition but not under shear stress. *Braz J Med Biol Res* 36: 1175–1178, 2003.
40. **Prabhu L, Upadhyaya P, Ram N, Nirodi CS, Sultana S, Vatsala PG, Mani SA, Rangarajan PN, Surolia A, and Padmanaban G.** A model for the transcriptional regulation of the CYP2B1/B2 gene in rat liver. *Proc Natl Acad Sci USA* 92: 9628–9632, 1995.
41. **Redmond EM, Cahill PA, and Sitzmann JV.** Flow-mediated regulation of G-protein expression in cocultured vascular smooth muscle and endothelial cells. *Arterioscler Thromb Vasc Biol* 18: 75–83, 1998.
42. **Resnick N, Collins T, Atkinson WJ, Bonthron DT, Dewey CF, and Gimbrone MA Jr.** Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci USA* 90: 4591–4595, 1993.
43. **Rieder MJ, Carmona R, Krieger JE, Pritchard KA Jr, and Greene AS.** Suppression of angiotensin-converting enzyme expression, and activity by shear stress. *Circ Res* 80: 312–319, 1997.
44. **Volpi S, Rabadan-Diehl C, Cawley N, and Aguilera G.** Transcriptional regulation of the pituitary vasopressin V1b receptor involves a GAGA-binding protein. *J Biol Chem* 277: 27829–27838, 2002.
45. **Wasserman SM, Mehraban F, Komuves LG, Yang RB, Tomlinson JE, Zhang Y, Spriggs F, and Topper JN.** Gene expression profile of human endothelial cells exposed to sustained fluid shear stress. *Physiol Genomics* 12: 13–23, 2002. First published November 5, 2002; 10.1152/physiolgenomics.00102.2002.
46. **Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, Meinhardt T, Pruss M, Reuter I, and Schacherer F.** TRANSFAC: an integrated system for gene expression. *Nucleic Acids Res* 28: 316–319, 2000.
47. **Wyse BD, Linas SL, and Thekkumkara TJ.** Functional role of a novel cis-acting element (GAGA box) in human type-1 angiotensin II receptor gene transcription. *J Mol Endocrinol* 25: 97–108, 2000.
48. **Yamamoto K, Korenaga R, Kamiya A, and Ando J.** Shear stress-dependent calcium entry via P2X4 receptors in human endothelial cells (Abstract). *FASEB J* 15: 412, 2000.
49. **Ziegler T, Bouzourene K, Harrison VJ, Brunner HR, and Hayoz D.** Influence of oscillatory and unidirectional flow environments on the expression of endothelin and nitric oxide synthase in cultured endothelial cells. *Arterioscler Thromb Vasc Biol* 18: 686–692, 1998.