NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS:

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Sp1 Sites Mediate Activation of the Plasminogen Activator Inhibitor-1 Promoter by Glucose in Vascular Smooth Muscle Cells*

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This study was designed to characterize the direct effects of hyperglycemia on plasminogen activator inhibitor-1 (PAI-1) expression in cultured vascular smooth muscle cells. Glucose induced dose- and time-dependent increases of PAI-1 mRNA expression in rat aortic smooth muscle (RASM) cells *in vitro*. Using a series of luciferase reporter gene constructs containing PAI-1 5′-flanking sequence (from −6.4 kilobase to −42 base pairs (bp)) transfected into RASM, we found that glucose (25 mM) consistently induced a 4-fold increase in luciferase activity, with the response localized to sequence between −85 and −42 bp. Mutagenesis of two putative Sp1-binding sites located in the region of interest essentially obliterated the glucose-response. Electrophoretic mobility shift assays with radiolabeled oligonucleotides containing the two putative Sp1-binding sites from PAI-1 promoter and nuclear extracts from RASM cells revealed that glucose treatment markedly changed the mobility pattern of the major protein-DNA complexes. Supershift assay showed that transcription factor Sp1 was present in the complexes under control and hyperglycemic conditions. These results suggest that glucose regulates PAI-1 gene expression in RASM cells through an effect on two adjacent Sp1 sites located between −85 and −42 bp of the PAI-1 5′-flanking region and that the release of a transcriptional repressor from the Sp1 complexes may explain the activation of the PAI-1 gene under high glucose conditions in RASM cells.

A number of different factors contribute to the development of atherosclerosis in patients with diabetes mellitus (1). Hyperglycemia has direct toxic effects on vascular tissue through the glycosylation of structural proteins (2). Hyperglycemia also appears to facilitate free radical formation and oxidative damage to the vascular wall (3). Conversely, the aggressive control of plasma glucose levels appears to delay the development of the common complications of diabetes in insulin-dependent diabetic patients (4). Many common risk factors also contribute to the atherogenic tendency in diabetes. These factors include abnormal lipoprotein metabolism, hypertension, and endothelial dysfunction. Recent studies have identified elevated levels of the fibrinolytic inhibitor, plasminogen activator inhibitor-1 (PAI-1),[1] in diabetic patients (5, 6). There is a developing appreciation for the role of PAI-1 plays in the pathogenesis in atherothrombotic disorders (7), and recent evidence suggests that increased PAI-1 production may be an important contributor to the development of vascular disease in diabetics (8). The increased production of PAI-1 seen in diabetic patients has been attributed directly to glucose, which increases PAI-1 production in cultured endothelium (9, 10), and by insulin and pro-insulin peptides (11), which induce PAI-1 production in cultured hepatocytes.

Recent studies have identified a cis-acting carbohydrate responsive element in the promoter region of the rat S14 gene (12). The carbohydrate responsive element consensus sequence has been deduced as 5′-CACGTGNNNGCC-3′ (13). This DNA motif has been shown to mediate glucose responsiveness when placed 5′ of genes that do not normally respond to glucose. Similar or identical DNA motifs have also been identified in other carbohydrate-responsive genes, including the genes for pyruvate kinase and the fatty acid synthase gene (13). Although it is clear that glucose can directly induce gene expression through a motif CACGTG that is related to the consensus binding site for the c-*myc* family of transcription factors, other glucose responsive elements have recently been identified, including Sp1 sites in the promoter II of the gene for acetyl-CoA carboxylase (14).

This study was designed to characterize the mechanism through which glucose regulates PAI-1 expression in vascular smooth muscle cells. Our findings indicate that glucose induces PAI-1 gene expression through two Sp1 sites located within 100 bases upstream of the transcription start site of PAI-1 gene.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was obtained from Hyclone Laboratory (Logan, UT). Tissue culture medium, transferrin, and ascorbic acid were from Life Technologies, Inc. (Gaithersburg, MD). Glucose, lactate, and insulin were from Sigma. Poly(dI-dC) was from Pharmacia Biotech (Uppsala, Sweden). Restriction enzymes and calf intestinal alkaline phosphatase were from Promega (Madison, WI). T4 DNA ligase, T4 DNA kinase, and Klenow fragment of DNA polymerase I were from New England Biolabs (Beverly, MA). Antibodies against Sp1 and Sp3 were from Santa Cruz Biotechnologies (Santa Cruz, CA) and monoclonal antibody against Rb protein was from Pharmingen (San Diego, CA). [γ-^{32}P]ATP (3000mCi/mmol), [α-^{32}P]dUTP, and [α-^{33}P]dATP were from NEN Life Science Products Inc. (Boston, MA).

Cell Culture—Rat aortic smooth muscle (RASM) cells were isolated as described previously (15). Cells were maintained in DMEM with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM HEPES. For these studies, RASM cells subcultured 10–16 times. The cells were identified as smooth muscle cells by both appearance and positive fibrillar staining with a smooth muscle specific α-ac-

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[1] The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; RASM, rat aortic smooth muscle; EMSA, electrophoretic mobility shift assay; bp, base pair(s); DMEM, Dulbecco's modified Eagle's medium.

TABLE I
Oligonucleotides used for mutagenesis

Wild-type PAI-I	Nucleotide sequence	Mutant PAI-I[a]
−85 to −63	5′-CAGTGAGTGGGTGGGGCTGGAAC-3′	
	AA TC	Mutant $Sp1_A$
−71 to −47	5′-GGCTGGAACATGAGTTCATCTATTT-3′	
	ATC A	Mutant P-box I
	AAGC G	Mutant P-box II
−53 to −30	5′-CATCTATTTCCTGCCCACATCTGGTA-3′	
	TA AT	Mutant $Sp1_B$

[a] The mutants were designed to introduce an Eco_{RI} restriction site in mutant $Sp1_A$, Cla I site in mutant P-box1, *Hin*dIII site in mutant P-box2, and *Xba*I in mutant $Sp1_B$ to allow identification by restriction digestion.

tin antibody (Boehringer Mannheim, Germany).

Plasmid Construction—PAI-1-luciferase reporter vectors were constructed by cloning various portions of the PAI-1 promoter into the pGL2-Basic luciferase vector (Promega, Madison, WI). Progressive deletions from the 5′ end of the human PAI-1 gene promoter fragments were constructed by using restriction endonuclease cleavage sites present in the PAI-1 promoter or using polymerase chain reaction methods to create fragments of PAI-1 promoter. All fragments had identical 3′ ends (*i.e.* the *Eco*RI site at position +71) and different 5′ ends. The following sites were used: for pGLuc 3.4k, the *Xba*I site at position −3.4 kilobase pairs, for pGLuc 1.5k, the *Kpn*I site at −1.5 kilobase pairs, and for pGLuc 884, the *Hin*dIII site at −884 bp. Specific oligonucleotide primers were used to synthesize smaller fragments using the polymerase chain reaction. The DNA fragments −107 bp, −85 bp, −54 bp, and −42 bp were treated with Klenow fragment to generate blunt ends, ligated to the pGL2 Basic luciferase reporter gene vector, and designated as follows: pGLuc 107, pGLuc 85, pGLuc 54, and pGLuc 42. The luciferase reporter gene vector (−6.4 kilobase pairs) of the PAI-1 promoter was kindly provided by Dr. David J. Loskutoff (Scripps Research Institute, La Jolla, CA).

Site-directed Mutagenesis—Base substitutions were made by oligonucleotide-mediated mutagenesis in three different regions identified as $Sp1_A$, $Sp1_B$, P-box I, and P-box II, corresponding to nucleotides −85 to −63, −53 to −30, and −71 to −47, respectively. The mutant oligonucleotides used as primers are listed in Table I together with the corresponding wild-type PAI-1 sequences. The mutations were either made with the Altered Sites *in vitro* mutagenesis kit (Promega) or carried out by the polymerase chain reaction method as described previously (16). Mutation accuracy was checked by restriction enzyme mapping and direct sequencing.

Transfection Assay—For transient transfection experiments, RASM cells were cultured in 35-mm 6-well plates at density of 1×10^5 cells/well. After cells reached 70–80% confluence, they were transfected using the DEAE-dextran method (17), with 1 μg of the PAI-1 pGL-2 construct and 0.8 μg of the plasmid pMVS-β-Gal, which contains the Lac Z gene under control of the Molony sarcoma virus long terminal repeat. The control plasmid has been examined on several occasions and has never demonstrated any response to glucose (data not shown). After overnight incubation, the media was replaced with serum starvation media (5.5 mM glucose, DMEM containing 5×10^{-7} M insulin, 5 μg/ml transferrin, and 0.2 mM ascorbic acid) for 24 h. This media has been demonstrated to maintain to smooth muscle cells in a quiescent, noncatabolic state for at least 72 h (18). The following morning, the cells were then kept in the glucose-deficient DMEM supplemented with either lactate (10 mM, final concentration) or glucose (25 mM, final concentration). After 48 h, the cells were harvested with reporter lysis buffer. The luciferase assays were performed according to the protocol of Promega. A 50-μl sample of the extract was used for determination of β-galactosidase activity by a colorimetric assay (19). The luciferase activity was corrected for β-galactosidase activity and is presented as corrected light units. To calculate relative induction, the corrected light unit of glucose-treated cells was divided from the corrected light unit of lactate-treated cells.

Northern Blot Analysis—For the Northern blot analysis, cells were grown in 100-mm tissue culture dishes. When the cells reached 80% confluence, the cells were then kept in the DMEM supplemented with different concentrations of glucose and glucosamine. Total cellular RNA isolation and Northern blot analysis were carried out as described (20).

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from RASM cells prepared as described previously (17). EMSAs were performed on 4% acrylamide gels with a buffer containing 50 mM Tris (pH 8.5), 0.38 M glycine, 2 mM EDTA, and 0.5 mM β-mercaptoethanol.

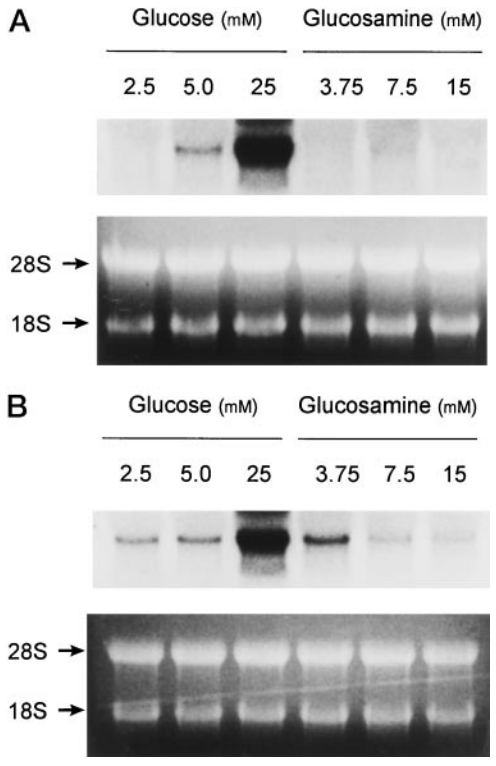


FIG. 1. **The effect of glucose and glucosamine on PAI-1 expression in cultured RASM cells.** *A*, 80% confluent cultures of RASM cells were exposed to glucose over the concentration range of 2.5–25 mM and glucosamine over concentration range of 3.75–15 mM in the presence of 5 mM glucose in DMEM without serum for 48 h. *B*, 80% confluent cultures of RASM cells were exposed to glucose over the concentration range of 2.5–25 mM and glucosamine over concentration range of 3.75–15 mM in the presence of 5 mM glucose in DMEM with 10% serum for 48 h.

Reactions were carried out in a 20-μl volume containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 6% glycerol, 1 μg of poly(dI-dC)·poly(dI-dC), and 3–6 μg of nuclear extract. The mixture was incubated at room temperature for 10 min and then 10,000 cpm of ^{32}P-labeled double-stranded oligonucleotides (0.1–0.6 ng). Following an additional incubation for 10 min at room temperature, the samples were electrophoresed at 200 V at 4 °C for 2 h. The gel was then dried and exposed to Kodak X-AR film. For competition experiments, 0.2–0.6 μg of unlabeled double-stranded oligonucleotides were added to the initial incubation mixture. For supershift experiments, 2 μg of anti-Sp1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was added to the reaction mixture and incubated for 2 h at 4 °C prior to adding the labeled probes. The following oligonucleotides covering sequences of 5′-flanking region of the PAI-1 gene were synthesized in the Molecular Biology Core facility of Vanderbilt University: −85/−63 sense, CAGTGAGTGGGT GGGGCTGGAAC; −85/−63 antisense, GTTCCAGCCCCACCCACTCACTG; −85/−63 mutated $Sp1_A$ sense, CAGTGAGTGAATTCGGCTGGAAC; −85/−63 mutated $Sp1_A$ antisense, GTTCCAGCCGAATTCACTCACTG; −53/−30 sense, CATCTATTTCCTGC CCACATCTGGTA; −53/−30 antisense, TACCAGATGTGGGCAGGAAATAGATG; −53/−30 mutated $Sp1_B$ sense, CATCTATTTCTAGATCACATCTGG; −53/−30 mutated $Sp1_B$ antisense, CCAGATGTGATCTAGAAATAGATG. Sp1 consensus oligonucleotides were purchased from Promega, and were comprised of the following nucleotide sequence: sense, ATTCGATCGGGGCGGGGCGAG; antisense, CTCGCCCCGCCCCGATCGAAT.

RESULTS

Effects of Glucose on Levels of PAI-1 mRNA in Rat and Bovine Aortic Smooth Muscle Cells—To determine whether glucose regulates PAI-1 gene expression, the relative expression of PAI-1 mRNA in RASM cells was measured by Northern blotting. In these experiments, confluent cultures of RASM cells were exposed to glucose over the concentration range of 2.5–25 mM in serum-free DMEM (Fig. 1*A*) or DMEM with 10% serum (Fig. 1*B*) for 48 h. Glucose resulted in a dose-dependent

increase in the expression of PAI-1 gene in RASM cells. No PAI-1 mRNA was detected in RASM cells treated with 2.5 mM glucose in the serum-free DMEM. On average, there was a 4.2 ± 2.2-fold increase in PAI-1 message induced by glucose (25 mM) compared with 5 mM glucose ($p = 0.0013$, $n = 6$). A time-dependent effect of glucose on PAI-1 gene expression in RASM cells was also demonstrated. The induction of PAI-1 mRNA expression produced by glucose is maximal at 72 h in RASM cells (Fig. 2). The specificity of this effect of glucose was studied by performing comparable studies using glucosamine, the hexosamine pathway metabolite of glucose. In the presence of 5 mM glucose, incremental concentrations of glucosamine failed to induce PAI-1 mRNA expression under serum-free conditions (Fig. 1*A*). Similarly, in serum-replete DMEM, increasing concentrations of glucosamine failed to induce PAI-1 mRNA expression in RASM cells (Fig. 1*B*).

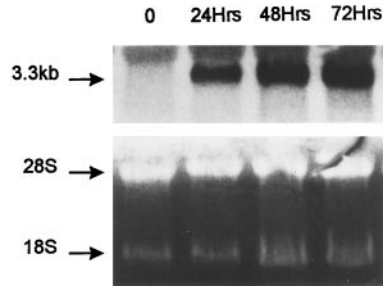


FIG. 2. **Time-dependent effect of glucose on PAI-1 expression in cultured RASM cells.** Confluent RASM cells were incubated in glucose-deficient DMEM supplemented with either 10 mM lactate or 25 mM glucose for 24–72 h. *kb*, kilobase pair(s).

Mapping of the Glucose-responsive Element in PAI-1 Promoter—The regulatory elements responsible for the induction of PAI-1 in RASM cells were localized with transient transfection assays. A schematic diagram of the PAI-1–1 5′-flanking region

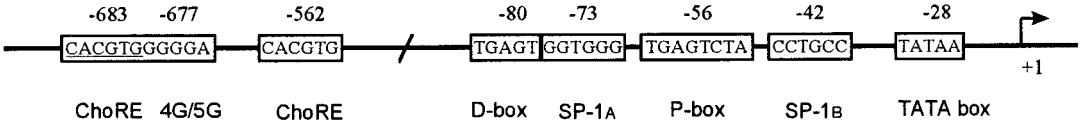
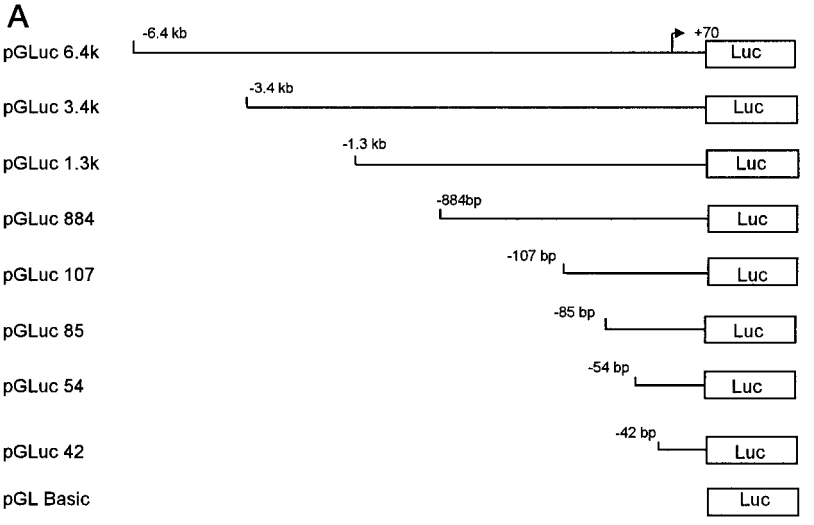


FIG. 3. **Schematic diagram of the upstream 5′-regulatory region of the PAI-1 gene showing the major described enhancer elements.** The TATA box and transcription start site are shown. Putative carbohydrate response elements are indicated by *ChoRE*.



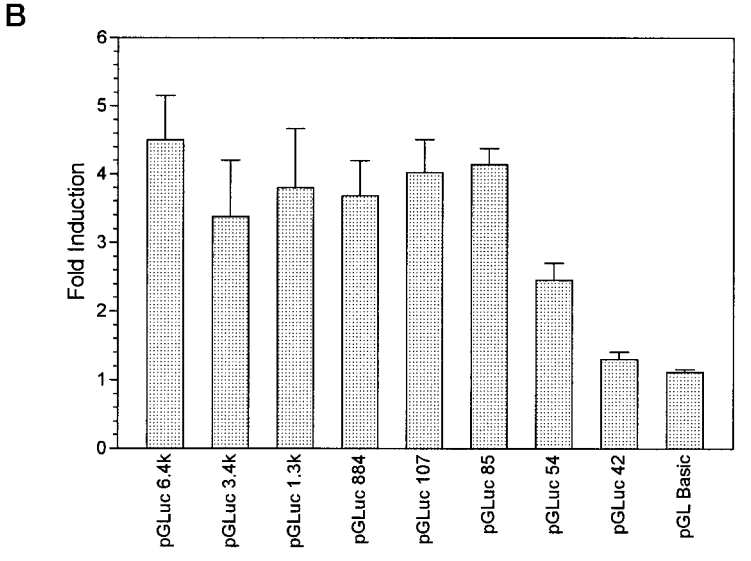


FIG. 4. *A*, schematic diagram of PAI-1 promoter deletion constructs used in transient transfection assays. Sequential deletions from the 5′ end were generated with the designation based on the number of base pairs upstream from the PAI-1 transcriptional start site as indicated. *B*, induction of luciferase activity with glucose in RASM cells transfected with PAI-1 promoter/luciferase reporter gene constructs. *Bars* represent the relative induction of luciferase activity after treatment with 25 mM glucose as compared with lactate-treated control cells. Results represent mean ± S.E. of at least five independent experiments.

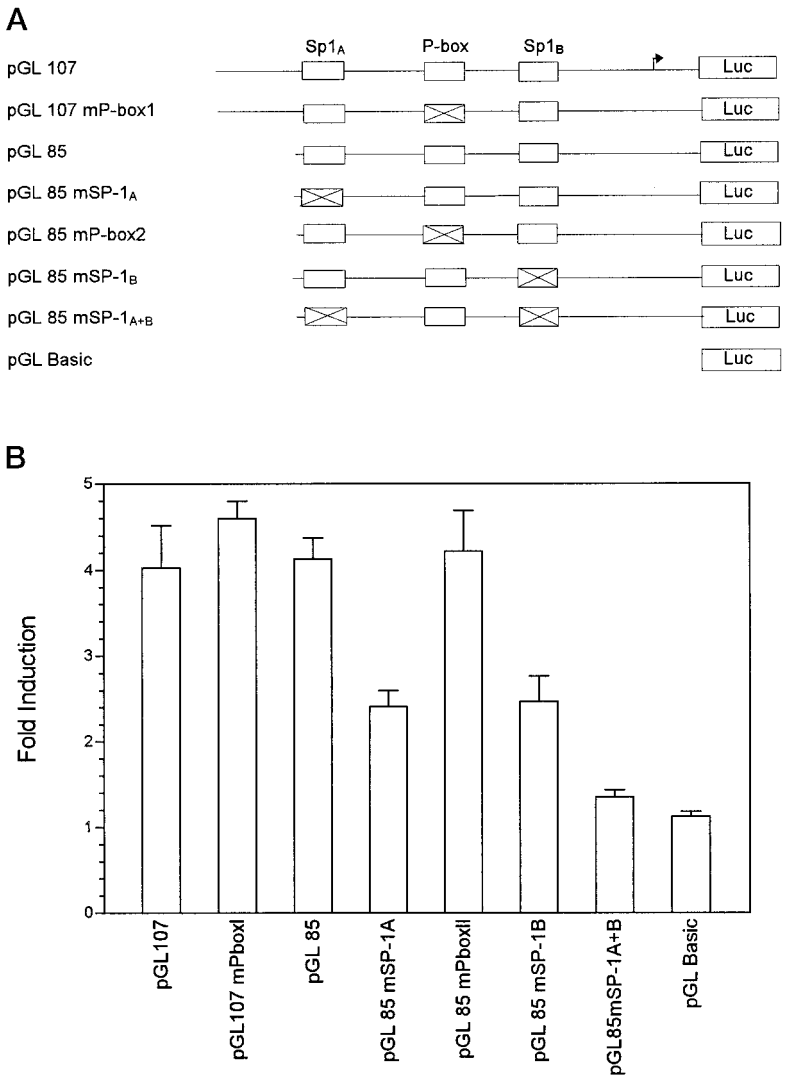


FIG. 5. *A,* schematic representation of the PAI-1 promoter mutation constructs used in transient transfection assays. Mutations in a particular element are indicated by a *X*. *B,* induction of luciferase activity with glucose in RASM cells transfected with PAI-1 promoter/luciferase reporter gene constructs. *Bars* represent the relative induction of luciferase activity after treatment with 25 mM glucose as compared with lactate-treated control cells. Results represent mean ± S.E. of at least five independent experiments.

and the described regulatory elements is shown in Fig. 3. Two putative sequences (CACGTG) identical to glucose response elements in the rat S14 gene (13) were found at sites −683 bp and −562 bp in the PAI-1 promoter. To localize the regulatory elements required for transcriptional activation of the PAI-1 gene by glucose, progressive 5′ promoter deletion constructs containing different portions of the PAI-1 promoter and 72 nucleotides of the PAI-1 5′-untranslated region were fused to a luciferase reporter gene. The constructs, schematically depicted in Fig. 4*A*, were transiently transfected into RASM cells and the luciferase activity was measured 48 h after lactate or glucose treatment of serum-starved RASM cells. To normalize for transfection efficiency, each construct was co-transfected with a constitutively expressed Molony sarcoma virus-β-galactosidase reporter construct. All experiments were repeated a minimum five times, and results are reported as mean ± S.E. In the presence of 25 mM glucose, there was a 3.7-fold increase in luciferase activity in RASM cells transfected with pGLuc884 construct, which includes both copies of the putative glucose response elements. To determine if the presence of the sequence motif CACGTG confers glucose responsiveness to a heterologous promoter, multiple copies of regions between −571 and −548 and between −698 and −664 of PAI-1 promoter were fused to the SV40 promoter-luciferase reporter gene vector as described above. These constructs were transfected into RASM cells and were not inducible by glucose (data not shown). As seen in Fig. 4*B*, constructs comprising 6.4k, 3.0k, 1.5k, 107, and 85 nucleotides of upstream sequences from the PAI-1 promoter all exhibited glucose responsiveness that averaged between 3.2–4.5-fold compared with controls. However, a substantial reduction in glucose responsiveness was observed in further truncated constructs, with pGLuc 54 exhibiting a nearly 50% reduction in glucose responsiveness. A further truncated construct, pGLuc 42, containing only PAI-1 TATA box, was not induced by glucose in comparison with the luciferase reporter construct pGL-2 Basic. These results indicate that the major sequence determinants of glucose responsiveness in the PAI-1 promoter reside between 85 and 42 nucleotides upstream from the transcription start site.

Mutagenesis of the Glucose-responsive Regions of PAI-1 Promoter—To further define the sequence(s) involved in the glucose responsiveness of PAI-1 promoter, site-directed mutagenesis was performed on the sequence between −85 and −42 in the pGLuc 107 and pGLuc 85 constructs. There are four recognized regulatory elements in the region, including two Sp1 sites, a P-box, and a D-box (Fig. 3). The Sp1 site is the binding site of the ubiquitously expressed transcription factor Sp1. The P box is similar in sequence to phorbol ester response elements and cAMP response elements. The D-box, a phorbol ester response elements-like sequence, has a weak affinity for transcription factor AP-1 (21, 22). In these experiments, four single mutants and different double mutants were constructed as shown in Fig. 5*A*. Mutations in P-box I or P-box II had no effect on the glucose responsiveness. Mutations in either $Sp1_A$ or

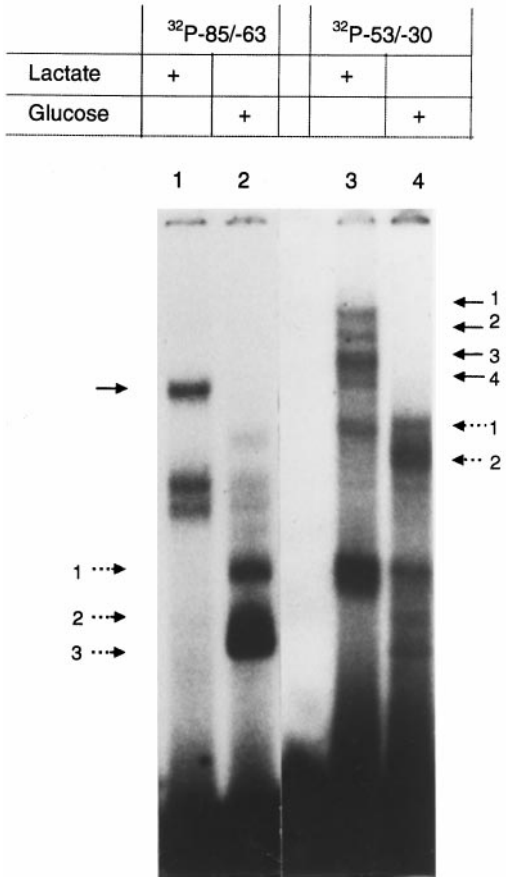


FIG. 6. **EMSA with RASM cell nuclear extracts.** The double-stranded oligonucleotides −85/−63 and −53/−30 of PAI-1 were end labeled and incubated with nuclear extracts from RASM cells grown in lactate- (*lanes 1* and *3*) or glucose-containing medium (*lanes 2* and *4*). *Solid arrows* indicate distinct bands identified under control (10 mM lactate) conditions and *broken arrows* indicate distinct bands present with high glucose.

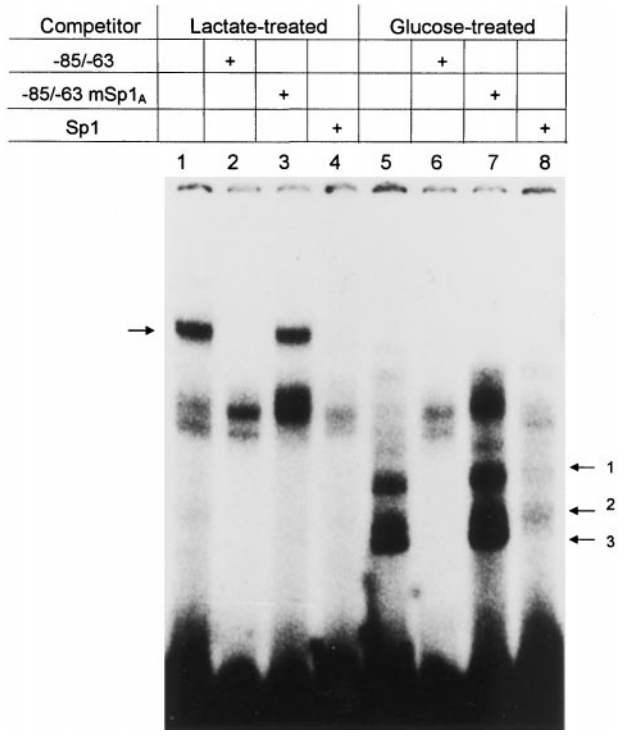


FIG. 7. **EMSA with ^{32}P-labeled −85/−63 oligonucleotide and RASM cell nuclear extract.** The double-stranded oligonucleotides −85/−63 of PAI-1 were end labeled and incubated with nuclear extracts from RASM cells grown in lactate- (*lanes 1–4*) or glucose-containing medium (*lanes 5–8*). *Lanes 1* and *5* were positive controls (no unlabeled competitor added). The unlabeled wide-type −85/−63 oligonucleotide (*lanes 2* and *6*), or −85/−63 oligonucleotide with mutant Sp1$_A$ site (*lanes 3* and *7*), or consensus Sp1 oligonucleotide (*lanes 4* and *8*) were added to the binding mixture in the mobility shift assay. *Arrows* indicate the specific bands.

Sp1$_B$ resulted in an approximately 50% decrease of glucose responsiveness compared with wild type pGLuc 85 construct. Mutations in Sp1$_A$ + Sp1$_B$ resulted in a loss of responsiveness to glucose (Fig. 5*B*). Thus, both Sp1 sites appear to be essential for glucose induction of PAI-1. These results support the involvement of the Sp1 transcriptional factor in the glucose activation of PAI-1 gene.

Identification of the Nuclear Factor(s) Involved in the Glucose Response—To test if transcription factor Sp1 binds to the site Sp1$_A$ and Sp1$_B$ in RASM cells, EMSAs were performed with RASM nuclear extracts prepared from either lactate-treated cells or glucose-treated cells and ^{32}P-labeled oligonucleotides consisting of Sp1$_A$ and Sp1$_B$ sequences spanning −85 to −63 and −53 to −30 of the PAI-1 promoter. As seen in Fig. 6, nuclear extracts from lactate-treated cells formed one unique complex with ^{32}P-labeled −85/−63 probe (*lane 1*) and four distinct complexes indicated by *arrows* on the *right* of Fig. 6 with the ^{32}P-labeled −53/−30 probe (*lane 3*). Extracts from glucose-treated cells formed different protein-DNA complexes with distinctly differing mobility. Three major complexes were formed with ^{32}P-labeled −85/−63 probe (*lane 2*) and two major complexes were formed with ^{32}P-labeled −53/−30 probe (*lane 4*). The DNA-protein complexes from glucose-treated cells migrated more rapidly than those from lactate-treated cells.

To determine the specificity of the DNA-protein complexes formed with the −85/−63 and −53/−30 probes, excess unlabeled consensus Sp1, wild-type, and mutant oligonucleotides were included in the binding reactions. The results of these competitive analyses on the formation of DNA-nuclear protein complexes are shown in Figs. 7 and 8. As seen in Fig. 7, one band from lactate-treated nuclear extract and three bands from glucose-treated nuclear extract are specific since competition with 100 molar excess unlabeled oligonucleotide (corresponding with sequence −85/−63) and consensus Sp1 oligonucleotides effectively prevented complex formation (*lanes 2, 4* and *6, 8*). The Sp1$_A$ site is critical for this binding, as unlabeled oligonucleotides with a mutated Sp1$_A$ site did not compete for the binding (*lanes 3* and *7*). In addition, excess unlabeled of wild-type −53/−30 oligonucleotide (containing Sp1$_B$) partially competed the bands (data not shown). As seen in Fig. 8, in either lactate-treated (*lanes 1–5*) or glucose-treated (*lanes 6–10*) nuclear extracts, several proteins bind specifically to the ^{32}P-labeled −53/−30 oligonucleotide containing Sp1$_B$ site (*lanes 1* and *6*). The specificity of these proteins is demonstrated by the fact that they were effectively competed by excess unlabeled wild-type −53/−30 oligonucleotide (*lanes 2* and *7*). The unlabeled oligonucleotide with a mutated Sp1$_B$ did not compete for binding (*lanes 3* and *8*). Interestingly, in the lactate-treated nuclear extract, the unlabeled wild-type −85/−63 oligonucleotide containing the Sp1$_A$ site reduced the apparent intensity of bands 1 and 3 (*lane 4*) while the consensus Sp1 oligonucleotide eliminated only band 1 (*lane 5*). In the glucose-treated nuclear extract, unlabeled wild-type −85/−63 oligonucleotide effectively eliminated bands 1 and 2 (*lane 9*) and the unlabeled consensus Sp1 oligonucleotide eliminated band 1 (*lane 10*).

Supershift assays were performed using antibody against Sp1, which has been reported to mediate the glucose response in the acetyl-CoA carboxylase gene (14). In the lactate-treated

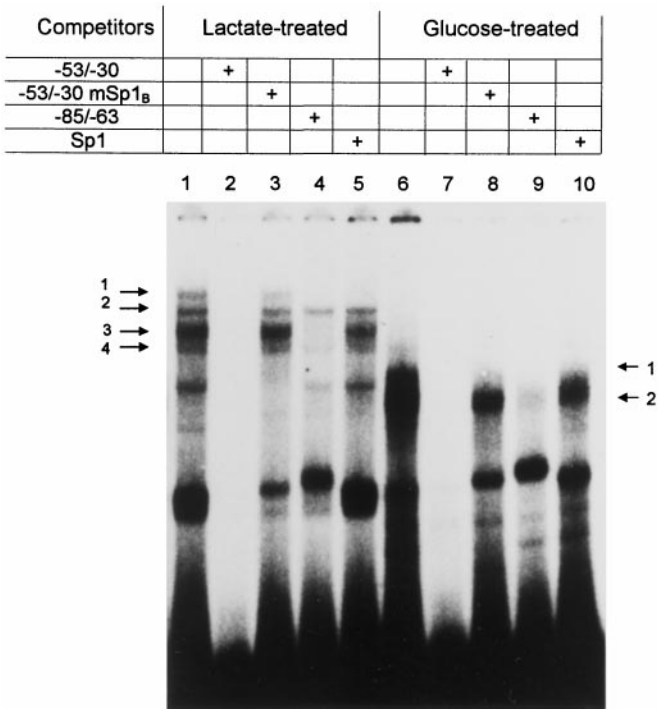


FIG. 8. **EMSA with ^{32}P-labeled −53/−30 oligonucleotide and RASM cell nuclear extract.** The double-stranded oligonucleotides −53/−30 of PAI-1 were end labeled and incubated with nuclear extracts from RASM cells grown in lactate- (*lanes 1–5*) or glucose-containing medium (*lanes 5–10*). *Lanes 1* and *6* were positive controls (no unlabeled competitor added). The unlabeled wide-type −53/−30 oligonucleotide (*lanes 2* and *7*), or −53/−30 oligonucleotide with mutant $Sp1_B$ site (*lanes 3* and *7*), or wild-type −85/−63 oligonucleotide (*lanes 4* and *9*), or consensus Sp1 oligonucleotide (*lanes 5* and *10*) was added to the binding mixture in the mobility shift assay. *Arrows* indicate the specific bands.

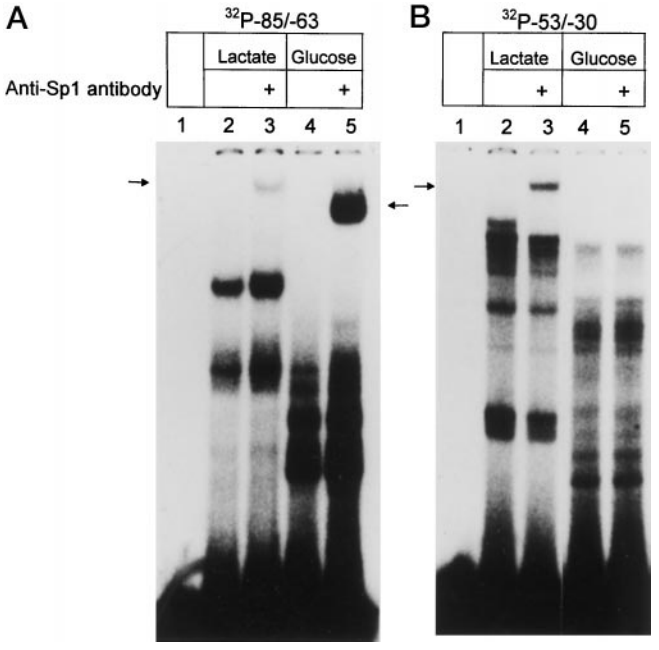


FIG. 9. **Supershift assays with Sp1 antibody.** *A*, the wild-type −85/−63 oligonucleotide was end labeled and added to the reaction mixture after the antibody reaction. The RASM cell nuclear extract was first incubated with (*lanes 3* and *5*) and without (*lanes 2* and *4*) anti-Sp1 antibody. *Lane 1* is a negative control (no nuclear extract added). *B*, the wild-type −53/−30 oligonucleotide was end labeled and added to the reaction mixture after the antibody reaction. The RASM cell nuclear extract was first incubated with (*lanes 3* and *5*) and without (*lanes 2* and *4*) anti-Sp1 antibody. *Lane 1* is a negative control (no nuclear extract added).

nuclear extract, the antibody against Sp1 yielded a novel band with reduced mobility using the ^{32}P-labeled −86/−63 probe (Fig. 9*A, lane 3*). The antibody against Sp1 also supershifted band 1 formed with the ^{32}P-labeled −53/−30 probe (Fig. 9*B, lane 3*). In contrast, in the glucose-treated nuclear extract, the antibody against Sp1 formed a larger, supershifted band with the ^{32}P-labeled −86/−63 probe (Fig. 9*A, lane 5*) and a very faint supershifted band with ^{32}P-labeled −53/−30 probe. Therefore, it appears that Sp1 is present in the complexes formed with the −85/−63 oligonucleotide under control and hyperglycemic conditions. Sp1 also appears to be present in complexes formed with the −53/−30 oligonucleotide under control conditions, but its presence under hyperglycemic conditions is uncertain. In similar experiments, a polyclonal antibody against Sp3 and a monoclonal antibody against Rb protein failed to induce a similar supershift (data not shown). These results indicate that Sp1 is one of the factors binding to the $Sp1_A$ and $Sp1_B$ sites located between nucleotides −85 and −30 of the PAI-1 promoter and suggest that the transcriptional complex or accessibility to antibody differs in the protein-DNA complexes bound to the two oligonucleotides.

DISCUSSION

Previous experimental studies have indicated that increased ambient glucose concentrations stimulate the expression of PAI-1 in cultured endothelial cells (9–11). The present studies extend this observation to include vascular smooth muscle cells. Increased glucose appears to induce dose-dependent and protracted increases in PAI-1 mRNA levels in cultured RASM cells. This effect is not merely a response to altered media osmolarity, as control experiments using equivalent concentrations of mannitol failed to produce similar effects (data not shown). Furthermore, glucosamine, a metabolite of glucose and the product of hexosamine pathway, did not mimic the effect of glucose in stimulating PAI-1 gene expression.

The recent description and identification of specific glucose-response elements in the regulatory regions of several genes prompted a systematic search for elements in the PAI-1 promoter that contribute to this response (14, 23, 24). Although sequence analysis suggested that candidate sequence motifs were present at −683 and −562 of the PAI-1 promoter, sequential deletion reporter constructs indicated that glucose responsiveness does not reside in these regions. Instead, glucose responsiveness was maintained in the reporter constructs down to a length of −85. Beyond that point, glucose responsiveness was lost in a stepwise manner following deletion to −54 and obliterated in deletion constructs down to −45. In this sequence reside two distinct Sp1 sites. Given previous reports that Sp1 sites mediate the glucose response in the acetyl-CoA carboxylase gene (14, 25), these sites were examined for their functional role in glucose responsiveness by examining the effects of site-specific mutations to these regions. Indeed, mutations in either Sp1 site of PAI-1 promoter independently reduced glucose responsiveness by approximately 50%, while constructs engineered to have mutations in both sites lost their glucose responsiveness. The specificity of these mutations is supported by the observation that mutation of the intervening P-box, located between the two Sp1 sites, failed to diminish glucose responsiveness. These findings confirm and extend previous observations that the glucose responsive region of the transforming growth factor-α promoter maps to a region including Sp1 sites (26, 27). Taken together, it appears that Sp1 sites may provide a mechanism for glucose responsiveness in vascular tissue.

The systematic analysis of the response of serially truncated or mutated reporter constructs merely identifies potential glu-

cose-responsive regions of the PAI-1 promoter. Although Sp1 sites are identified, this does not establish that the ubiquitous transcription factor Sp1 is responsible for this effect. However, EMSA studies confirmed that the electrophoretic mobility of oligonucleotides comprised of these regions of interest was altered by increased glucose concentrations. Furthermore, immunological analyses of these complexes suggested that authentic Sp1 was present under control conditions and remained associated to at least one of the labeled Sp1 sites in the presence of increased glucose. Taken together, these findings suggest the possibility that Sp1 is bound to the putative Sp1 sites under normal conditions, but that transcription is suppressed by the presence of a bound repressor. In the presence of increased glucose, the repressor molecules are released, leaving Sp1 bound to the Sp1 sites, and transcription ensues. Ample precedent exists to permit this mechanism to be entertained as a potential explanation for the effects of glucose described in this study and in fact, a similar mechanism has recently been described through which Sp1 mediates induced expression of the acetyl carboxylase gene (14). Alternatively, glucose has been reported to induce gene expression by promoting the binding of transcriptional activators (14, 24, 28). Although the present findings are not entirely consistent with this possibility, it cannot be completely excluded.

We have suggested that hyperglycemia promotes the displacement of a transcriptional repressor present in the Sp1 transcriptional complex. However, the identity of this repressor protein or proteins is unknown at present. Studies with antibodies against the retinoblastoma gene product and against the related protein Sp3 have failed to establish the presence of these proteins in EMSA studies. Further studies are now underway to identify potential negative and positive co-factors in the Sp1 regulatory complex localized to the Sp1 sites in the PAI-1 promoter.

As atherosclerotic lesions progress in terms of severity (Stage IV and beyond), there is increased deposition of fibrin, fibrinogen, and collagen in areas of advanced atherosclerosis (29). Advanced lesions are also characterized by smooth muscle cell invasion and proliferation. The fibrinolytic system, *i.e.* plasmin, is primarily responsible for the proteolytic degradation of fibrin and fibrinogen. Furthermore, plasmin is indirectly responsible for the dismantling of excess collagen by virtue of the role it plays in the activation of matrix metalloproteinases (30). Plasmin also plays a role in regulating the smooth muscle cell content of atherosclerotic lesions through its role in activating latent transforming growth factor-β (31), which has potent antimigratory and antiproliferative effects on vascular smooth muscle cells. Thus, it is quite evident that plasmin plays a multifaceted housekeeping role in the vessel wall, and that fibrinolytic activity likely retards the progression of atherosclerotic lesions. This view is supported by recent studies indicating that plasminogen deficiency accelerated the development of atherosclerotic lesions in ApoE knockout mice (32). Apart from its effects on intraluminal fibrinolytic balance, PAI-1 is topologically distributed to influence fibrinolytic function in the vessel wall. Several studies have now confirmed the presence of increased amounts of PAI-1 in human atherosclerotic plaques (7, 33, 34). The presence of excess PAI-1 in atherosclerotic lesions reduces local plasmin generation (35). In such a milieu, a relative paucity of plasmin production likely reduces matrix remodeling capacity. Furthermore, since transforming growth factor-β has potent antiproliferative effects on vascular smooth muscle cells (36), a reduction in the plasmin-dependent activation of this critical cytokine likely exerts a permissive effect on the growth of an atherosclerotic lesion. Thus, increased local production of PAI-1 likely contributes to the progression of atherosclerotic lesions, and PAI-1 expression appears to be regulated by glucose in vascular tissues. This relationship may play an important role in the pathogenesis of atherothrombotic disease in diabetes.

In conclusion, these studies indicate that hyperglycemia directly stimulates PAI-1 expression in cultured VSMC. This response appears to be localized to two independent Sp1 sites just upstream from the transcription start site of PAI-1. The present findings suggest that glucose induces this effect on PAI-1 transcription by dislodging an unidentified transcriptional repressor from the Sp1 complex. The ability of glucose to stimulate directly the expression of PAI-1 in vascular tissue may have important clinical ramifications. Local overexpression of PAI-1 in the blood vessel wall would be expected to contribute to the development of atherosclerosis and fibrosis. The development of diffuse and severe vascular disease in diabetes is an all too common manifestation of the disease, and is responsible for a great deal of the excess cardiovascular morbidity and mortality seen in this disorder. Improved glucose control would be expected to result in reduced vascular PAI-1 expression, which in turn may contribute to a reduction in the development of vascular disease and its complications.

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