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Early Growth Response Factor-1 Mediates Insulin-Inducible Vascular Endothelial Cell Proliferation and Regrowth After Injury

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Abstract Hyperinsulinemia in diabetes mellitus is a significant risk factor in the development of atherosclerosis and early restenosis after balloon angioplasty. These manifestations could be mediated by the ability of insulin to potentiate the cellular proliferative and reparative response of vascular cell types to local stimuli. Here we demonstrate that insulin stimulates DNA synthesis in aortic endothelial cells. Reverse transcription-polymerase chain reaction and Northern blotting revealed that insulin induces the expression and transcriptional activity of the immediate early gene and zinc finger transcription protein, early growth response factor-1 (Egr-1). Western immunoblot analysis revealed that insulin-inducible Egr-1 expression was inhibited using phosphorothioate-specific antisense oligonucleotides targeting Egr-1 mRNA. These agents blocked endothelial cell DNA synthesis stimulated by insulin in a dose-dependent manner and inhibited the capacity of insulin to potentiate the reparative response of endothelial cells to mechanical injury in vitro. These oligonucleotides also attenuated wound repair in smooth muscle cells. DNA synthesis induced by insulin was suppressed by inhibitors of two upstream activators of Egr-1, extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-phosphate (PI 3-K), whereas p38 kinase inhibitors had no effect. These present findings demonstrate that insulin-inducible DNA synthesis and repair after injury are processes critically dependent upon the activation of Egr-1. Additionally, they implicate this transcription factor as a potential target for the inhibition of restenosis in diabetics. J. Cell. Biochem. 81:523–534, 2001.

Key words: Egr-1; insulin; endothelial cells; proliferation; wound repair

Diabetes mellitus is a major independent risk factor in the pathogenesis of atherosclerosis. Morbidity and mortality from cardiovascular disease is two- to threefold greater in diabetics [Kannel and McGee, 1979; Stamler et al., 1993]. Diabetics have greater risk of acute myocardial infarction [Lundberg et al., 1997], in-hospital mortality [Klamann et al., 2000] and require additional revascularization procedures after percutaneous transluminal coronary angioplasty due to restenosis [Stein et al., 1995; Kip et al., 1996]. Calcification in coronary atherosclerosis is more frequent in women diabetics and is associated with plaque rupture [Burke et al., 2000]. Atherosclerosis accounts for approximately 80% of all deaths from diabetes; the vast majority of these are attributable to coronary artery disease [Garber, 1998].

Endothelial cells form a continuous monolayer which lines the entire cardiovascular system. Activation of these normally quiescent cells has long been implicated as an early event in the pathogenesis of atherosclerosis [Ross, 1993]. Dysfunctional endothelium triggers platelet aggregation, adhesion and transmigration by circulating leukocytes, chemotaxis and proliferation by underlying smooth muscle cells, and alters vascular tone. A positive correlation in the plasma of diabetics between free N-terminal fibronectin and von Willebrand factor provided supportive evidence for a link between endothelial dysfunction in diabetes mellitus in humans [Skrha et al., 1990]. Insulin activates endothelial superoxide anion pro-

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duction in exogenously hyperinsulinemic rats [Kashiwagi et al., 1999], cholesterol ester accumulation in the arteries of normal rats [Abe et al., 1996] and potentiates smooth muscle cell proliferation after endothelial injury in hyperinsulinemic obese Zucker rats [Ridray et al., 1994]. Insulin stimulates endothelin-1 expression and secretion [Hu et al., 1993], collagen IV biosynthesis [Hayakawa et al.,1994] and apoptosis [Du et al., 1998] of cultured endothelial cells. Insulin is also a mitogen and chemoattractant for cultured vascular smooth muscle cells [Stout, 1991].

The insulin receptor is a heterotetrameric glycoprotein comprising two extracellular ligand-binding α-subunits and two transmembrane β-subunits which bear inherent tyrosine protein kinases. These kinases autophosphorylate the receptor and phosphorylate endogenous substrates, such as insulin receptor substrate-1, that activate a number of cytoplasmic signaling proteins, including phoshatidylinositol 3-kinase and mitogen-activated protein kinases that, in turn, influence transcription of insulin-responsive genes [White and Kahn, 1994]. Presently little is known about the functional roles of specific transcription factors in the responsiveness of vascular endothelial cells to insulin.

Early growth response factor-1 (Egr-1, also known as zif268, krox24, TIS8) is the product of an immediate early gene and a prototypical member of the zinc finger family of transcriptional regulators [Gashler and Sukhatme, 1995]. Egr-1 binds to the promoters of a spectrum of genes implicated in the pathogenesis of atherosclerosis and restenosis. These include the platelet-derived growth factor (PDGF) A-chain [Khachigian et al., 1995], PDGF-B [Khachigian et al., 1996], transforming growth factor-β_1 [Liu et al., 1996, 1998], fibroblast growth factor-2 (FGF-2) [Hu and Levin, 1994; Biesiada et al., 1996], membrane type 1 matrix metalloproteinase [Haas et al., 1999], tissue factor [Cui et al., 1996] and intercellular adhesion molecule-1 [Maltzman et al., 1996]. Egr-1 has also been localised to endothelial cells and smooth muscle cells in human atherosclerotic plaques [McCaffrey et al., 2000]. Suppression of Egr-1gene induction using sequence-specific catalytic DNA inhibits intimal thickening in the rat carotid artery following balloon angioplasty [Santiago et al., 1999]. Here we explored the hypothesis that the pro-atherogenic effects of insulin are mediated, at least in part, through this transcription factor. We demonstrate that insulin stimulates endothelial cell proliferation and regrowth following mechanical injury and that both these processes are critically dependent upon the activation of Egr-1.

MATERIALS AND METHODS

Oligonucleotides and Chemicals

Phosphorothioate-linked antisense oligonucleotides directed against the region comprising the translational start site of Egr-1 mRNA were synthesized commercially (Genset Pacific) and purified by high performance liquid chromatography. The target sequence of AS2 (5′-CsTsTsGsGsCsCsGsCsTsGsCsCsAsT-3′) is conserved in mouse, rat, and human Egr-1 mRNA. For control purposes, we used AS2C (5′-GsCsAsCsTsTsCsTsGsCsTsGsTsCsC-3′), a size-matched phosphorothioate-linked counterpart of AS2 with a similar base composition. Phorbol-12-myristrate 13-acetate (PMA) and fibroblast growth factor-2 were purchased from Sigma-Aldrich. Wortmannin and PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1 benzopyrane4-one) were obtained from Calbiochem.

Cell Culture

Bovine aortic endothelial cells and rat aortic smooth muscle cells were obtained from Cell Applications, Inc. and used between passages 5–9. The endothelial cells were grown in Dulbecco's modified Eagles' medium (Life Technologies, Gaithersburg, MD), pH 7.4, containing 10% fetal bovine serum supplemented with 50 μg/ml streptomycin and 50 IU/ml penicillin. The smooth muscle cells were grown in Waymouth's MB752/1 medium (Life Technologies) supplemented with 30 μg/ml L-glutamine and antibiotics. The cells were routinely passaged with trypsin/EDTA and maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Transient Transfection Analysis and CAT Assay

The endothelial cells were grown to 60–70% confluence in 100 mm dishes and transiently transfected with 10 μg of the indicated chloramphenicol acetyl transferase (CAT)-based promoter reporter construct using FuGENE6 (Roche). The cells were rendered growth-quiescent by incubation 48 h in 0.25% FBS, and stimulated with various agonists for 24 h

prior to harvest and assessment of CAT activity. CAT activity was measured and normalized to the concentration of protein in the lysates (determined by Biorad Protein Assay) as previously described [Khachigian et al., 1999].

Northern Blot Analysis

Total RNA (12 μg/well) of growth-arrested endothelial cells (prepared using TRIzol Reagent (Life Technologies) in accordance with the manufacturer's instructions) previously exposed to various agonists for 1 h was resolved by electrophoresis on denaturing 1% agarose-formaldehyde gels. Following transfer overnight to Hybond- N^+ nylon membranes (Amersham), the blots were hybridized with ^{32}P-labeled Egr-1 cDNA prepared using the Nick Translation Kit overnight (Roche). The membranes were washed and radioactivity visualized by autoradiography as previously described [Khachigian et al., 1995].

RT-PCR

Reverse transcription was performed with 8 μg of total RNA using M-MLV reverse transcriptase. Egr-1 cDNA was amplified (334 bp product [Delbridge and Khachigian, 1997]) using Taq polymerase by heating for 1 min at 94°C, and cycling through 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following 30 cycles, a 5-min extension at 72°C was carried out. Samples were electrophoresed on 1.5% agarose gel containing ethidium bromide and photographed under ultraviolet illumination. β-actin amplification (690 bp product) was performed essentially as above. The sequences of the primers were: Egr-1 forward primer (5′-GCA CCC AAC AGT GGC AAC-3′), Egr-1 reverse primer (5′-GGG ATC ATG GGA ACC TGG-3′), β-actin forward primer (5′-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3′), and β-actin reverse primer (5′-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3′).

Antisense Oligonucleotide Delivery and Western Blot Analysis

Growth-arrested cells in 100 mm dishes were incubated with the indicated oligonucleotides 24 and 48 h after the initial change of medium. When oligonucleotide was added a second time, the cells were incubated with various concentrations of insulin and harvested 1 h subsequently. The cells were washed in cold phosphate-buffered saline (PBS), pH 7.4, and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μg/ml leupeptin, 1% aprotinin, 2 μM PMSF). Lysates were resolved by electrophoresis on 8% denaturing SDS-polyacrylamide gels, transferred to PDVF nylon membranes (NEN-DuPont), blocked with skim milk powder, then incubated with polyclonal antibodies to Egr-1 (Santa Cruz Biotechnology, Inc.) and monoclonal horseradish peroxidase-linked mouse anti-rabbit Ig secondary antibodies followed by chemiluminescent detection (NEN-DuPont).

^{3}H-Thymidine Incorporation Into DNA

Growth-arrested endothelial cells at 90% confluence in 96-well plates were incubated twice with the oligonucleotides prior to the addition of insulin. When signaling inhibitors (PD98059, SB202190, wortmannin) were used in experiments, these agents were added 2 h before the addition of insulin. After 18 h of exposure to insulin, the cells were pulsed with 200,000 cpm/well of methyl-^{3}H thymidine (NEN-DuPont) for 6 h. Lysates were prepared by washing first in cold PBS, pH 7.4, then fixing with cold 10% trichloroacetic acid, washing with cold ethanol and solubilizing in 0.1 M NaOH. ^{3}H-Thymidine in the lysates was quantitated with ACSII scintillant using β-scintillation counter (Packard).

In Vitro Injury

Growth-arrested cells at 90% confluence were incubated with antisense oligonucleotides and insulin at various concentrations as described above, then were scraped by drawing a sterile wooden toothpick across the monolayer [Khachigian et al., 1996]. Following 48–72 h, the cells were fixed in 4% formalin, stained with hematoxylin/eosin then photographed.

RESULTS

Insulin, but not Glucose, Stimulates Egr-1 Activity in Vascular Endothelial Cells

Hyperglycemia has been linked with endothelial dysfunction [Cosentino and Luscher, 1998] and contributes to the pathogenesis of atherosclerotic lesions in animal models [Stout, 1991]. High glucose may activate normally quiescent vascular endothelium by stimulating

mitogen-activated protein (MAP) kinase activity and the expression of immediate-early genes [Frodin et al., 1995; Kang et al., 1999]. These signaling and transcriptional events may, in turn, induce the expression of other genes whose products then alter endothelial phenotype and facilitate the development of lesions. To determine the effect of glucose on Egr-1 activity in vascular endothelial cells, we performed transient transfection analysis in endothelial cells transfected with pEBS1^{3}foscat, a chloramphenical acetyltransferase (CAT)-based reporter vector driven by three high-affinity Egr-1 binding sites placed upstream of the c-*fos* TATA box [Gashler et al., 1993]. Exposure of growth-arrested endothelial cells to various concentrations of glucose (5–30 mM) over 24 h did not increase Egr-1 binding activity (Fig. 1). However, Egr-1 binding activity did increase in cells exposed to insulin (100 nM) (Fig. 1). Reporter activity also increased upon incubation with FGF-2, a known inducer of Egr-1 transcription and binding activity in vascular endothelial cells [Santiago et al., 1999] (Fig. 1). Insulin-inducible Egr-1 activity has hitherto not been demonstrated in vascular endothelial cells.

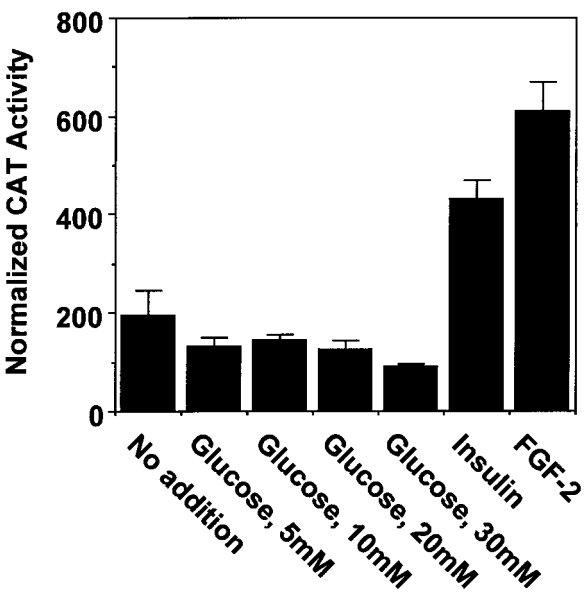

Fig. 1. Insulin stimulates Egr-1-dependent gene expression in vascular endothelial cells. Growth-arrested bovine aortic endothelial cells previously transfected with pEBS1^{3}foscat using FuGENE6 were incubated with D-glucose (5–30 mM), insulin (100 nM) or FGF-2 (25 ng/ml) as indicated for 24 h prior to preparation of cell lysates. CAT activity was normalized to the concentration of protein in the lysates.

Insulin and FGF-2 Induce Egr-1 mRNA Expression in Vascular Endothelial Cells

The preceding findings using reporter gene analysis provided evidence for increased Egr-1 expression in endothelial cells exposed to insulin. We next used reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis to demonstrate directly the capacity of insulin to increase levels of Egr-1 mRNA. RT-PCR revealed that Egr-1 is weakly expressed in growth-quiescent endothelial cells (Fig. 2A). Insulin, like FGF-2, increased Egr-1 expression within 1 h of exposure to the agonist (Fig. 2A). In contrast, levels of β-actin mRNA were unchanged (Fig. 2A). Northern blot analysis confirmed these qualitative data by demonstrating that insulin, FGF-2, and phorbol 12-myristate 13-acetate (PMA), a second potent inducer of Egr-1 expression [Khachigian et al., 1995] elevated steady-state Egr-1 mRNA levels within 1 h without increasing levels of ribosomal 28S and 18S mRNA (Fig. 2B).

Insulin-Stimulated Egr-1 Protein Synthesis in Endothelial Cells is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA

To reconcile our demonstration of insulin-induced Egr-1 mRNA expression (Fig. 2) with the binding activity of the transcription factor (Fig. 1), we performed Western immunoblot analysis using polyclonal antibodies directed against Egr-1 protein. Insulin (at 100 and 500 nM) induced Egr-1 protein synthesis in growth-arrested endothelial cells within 1 h (Fig. 3). These findings, taken together, demonstrate that insulin elevates Egr-1 mRNA (Fig. 2), protein (Fig. 3) and binding activity (Fig. 1) in vascular endothelial cells.

We recently developed phosphorothioate-based antisense oligonucleotides targeting the translational start site in Egr-1 mRNA [Santiago et al., 1999a, 1999b]. These oligonucleotides lack phosphorothioate G-quartet sequences that have been associated with non-specific biological activity [Stein, 1997]. Western blot analysis revealed that prior incubation of growth-arrested endothelial cells with 0.8 μM antisense Egr-1 oligonucleotides (AS2) inhibited insulin-inducible Egr-1 protein synthesis (Fig. 3), despite equal loading of protein (Fig. 3). The lack of attenuation in insulin-inducible Egr-1 protein following exposure of the cells to an identical concentration of AS2C (Fig. 3), de-

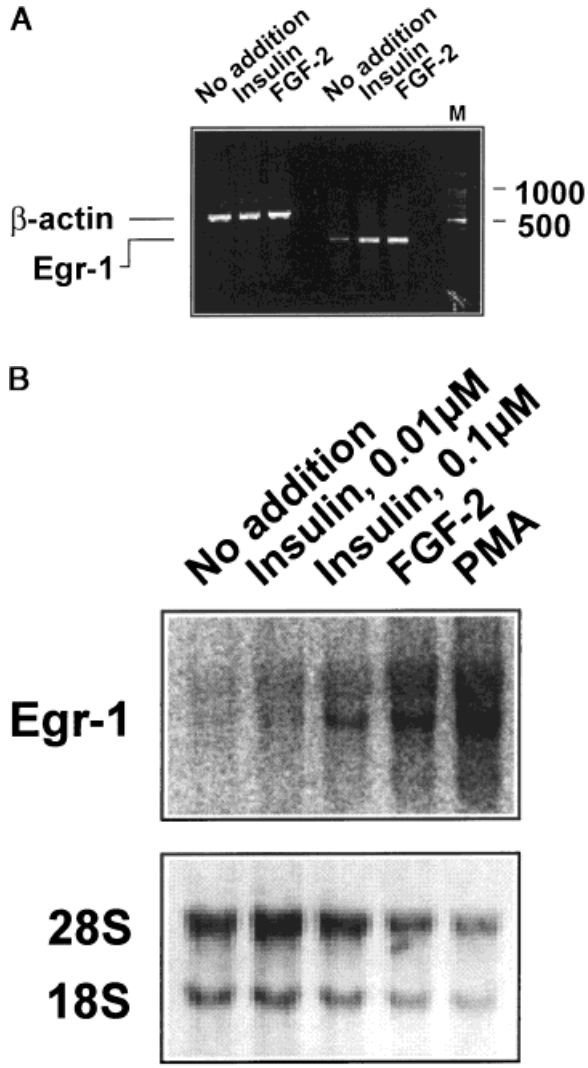

Fig. 2. Insulin induces Egr-1 mRNA expression in vascular endothelial cells. **A**. RT-PCR. Growth-quiescent endothelial cells were incubated with insulin (100 nM) or FGF-2 (25 ng/ml) for 1 h prior to the preparation of total RNA. PCR was performed on M-MLV reverse transcribed cDNA. The expected fragment sizes of amplified Egr-1 and β-actin cDNA were 334 bp and 690 bp, respectively. **B**. Northern blot analysis using total RNA prepared from growth-arrested endothelial cells exposed to insulin (10 or 100 nM), FGF-2 (25 ng/ml) or PMA (100 ng/ml). The ethidium bromide-stained gel showing 28S and 18S RNA appears in the lower panel.

monstrates the sequence-specific inhibitory effect of the antisense Egr-1 oligonucleotides.

Insulin Stimulates Endothelial Cell DNA Synthesis Which is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA

These oligonucleotides, which attenuate the induction of Egr-1 protein (Fig. 3), were used in ^{3}H-thymidine incorporation assays to determine the involvement of Egr-1 in insulin-indu-

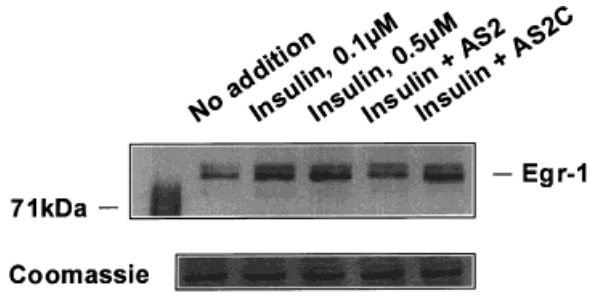

Fig. 3. Egr-1 protein levels increase in endothelial cells exposed to insulin and are inhibited by antisense oligonucleotides targeting Egr-1. Growth-arrested endothelial cells were incubated with 0.8 μM of oligonucleotides AS2 or AS2C prior to the addition of insulin (500 nM) for 1 h. Lysates were resolved by denaturing electrophoresis, transfered to nylon membranes and incubated with polyclonal antibodies directed against Egr-1. Binding was visualized by chemiluminescent detection. The Coomassie Blue stained gel indicates equal protein loading.

cible DNA synthesis. This assay evaluates ^{3}H-thymidine uptake into DNA precipitable with trichloroacetic acid (TCA) [Khachigian and Chesterman, 1992]. In initial experiments, growth-arrested endothelial cells exposed to insulin (100 nM) increased the extent of DNA synthesis by 100%, whereas 500 nM insulin caused a 200% increase in DNA synthesis (Fig. 4A). These findings using bovine aortic endothelial cells (BAEC), contrast with earlier observations indicating the inability of insulin to increase DNA synthesis in this cell type [Taggart and Stout, 1980; King et al., 1983] and in human umbilical vein endothelial cells [King et al., 1983].

We next determined the effect of AS2 and AS2C on insulin-inducible endothelial DNA synthesis. In the absence of added insulin, AS2 (0.8 μM) inhibited basal endothelial DNA synthesis facilitated by low concentrations of serum (0.25%, v/v) (Fig. 4B). In contrast, AS2 (0.8 μM) or a third oligonucleotide, E3 (0.8 μM), a size-matched phosphorothioate directed toward another region of Egr-1 mRNA [Santiago et al., 1999] had little effect on basal DNA synthesis (Fig. 4B). Furthermore, unlike AS2 and E3, AS2 significantly inhibited DNA synthesis inducible by insulin (500 and 1000 nM) (Fig. 4B). To demonstrate concentration-dependent inhibition of DNA synthesis, we incubated the endothelial cells with 0.4 μM as well as 0.8 μM of Egr-1 oligonucleotide. Since this lower concentration of AS2 inhibited ^{3}H-thymidine incorporation less effectively (compare to AS2C) indicates dose-dependent and sequence-specific inhibition by the antisense

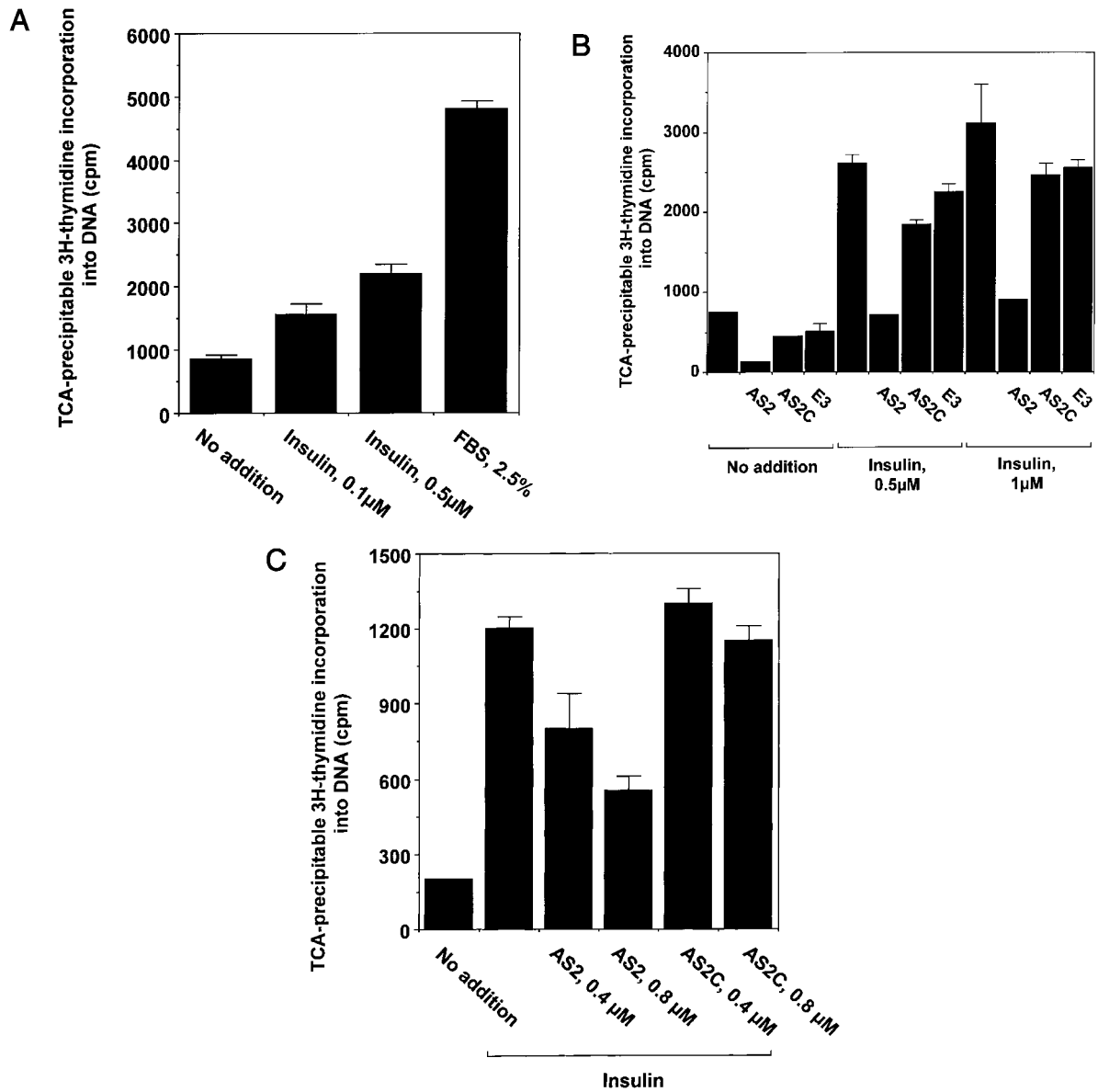

Fig. 4. Insulin-induced DNA synthesis in aortic endothelial cells is blocked by antisense oligonucleotides targeting Egr-1. **A.** Insulin stimulates DNA synthesis. Growth-arrested endothelial cells were incubated with insulin (100 or 500 nM) or FBS (2.5%) for 18 h prior to ^{3}H-thymidine pulse for a further 6 h. **B.** Antisense Egr-1 oligonucleotides inhibit insulin-inducible DNA synthesis. Endothelial cells were incubated with 0.8 μM of either AS2, AS2C or E3 prior to exposure to insulin (500 or 1000 nM) for 18 h and ^{3}H-thymidine pulse for 6 h. **C.** Dose-dependent inhibition of insulin-inducible DNA synthesis. DNA synthesis stimulated by insulin (500 nM) was assessed in endothelial cells incubated with 0.4 or 0.8 μM of AS2 or AS2C. TCA-precipitable ^{3}H-thymidine incorporation into DNA was assessed using a β-scintillation counter.

Egr-1 oligonucleotide (Fig. 4C). These findings thus demonstrate the requirement for Egr-1 protein in endothelial cell DNA synthesis inducible by insulin.

Insulin-Stimulated DNA Synthesis in Endothelial Cells is Inhibited by PD98059 and Wortmannin, but not by SB202190

Inducible Egr-1 transcription is governed by the activity of extracellular signal-regulated kinase (ERK) [Santiago et al., 1999] which phosphorylates factors at serum response elements in the Egr-1 promoter [Gashler and Sukhatme, 1995]. Since little is known about signaling pathways mediating insulin-inducible proliferation of vascular endothelial cells, we determined the relevance of MEK/ERK in this process using the specific MEK/ERK inhibitor, PD98059. This compound (at 10 and 30 μM) inhibited insulin-inducible DNA synth-

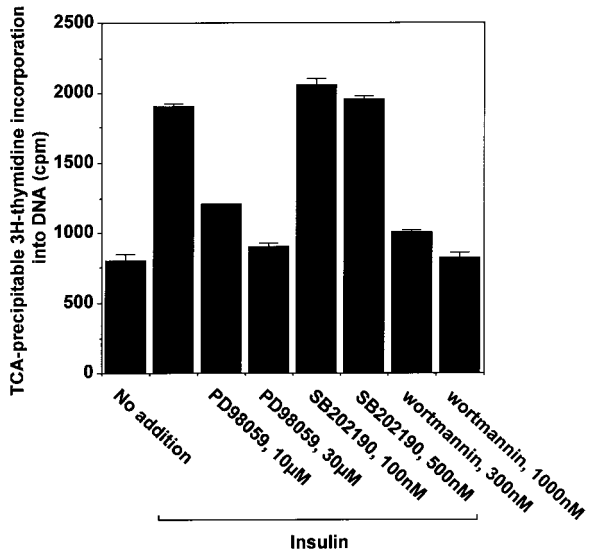

Fig. 5. Insulin-inducible DNA synthesis in cultured aortic endothelial cells is MEK/ERK-dependent. Growth-quiescent endothelial cells were preincubated for 2 h with either PD98059 (10 or 30 μM), SB202190 (100 or 500 nM) or wortmannin (300 or 1000 nM) prior to the addition of insulin (500 nM) for 18 h and ^{3}H-thymidine pulse. TCA-precipitable ^{3}H-thymidine incorporation into DNA was assessed using a β-scintillation counter.

esis in a dose-dependent manner (Fig. 5). Likewise, wortmannin (0.3 and 1 μM), the phosphatidylinositol 3-kinase inhibitor which also inhibits c-Jun N-terminal kinase (JNK) [Day et al., 1999; Ishizuka et al., 1999; Kumahara et al., 1999], ERK [Barry et al., 1999] and p38 kinase [Barry et al., 1999] inhibited DNA synthesis in a dose-dependent manner (Fig. 5). In contrast, SB202190 (100 and 500 nM), a specific p38 kinase inhibitor failed to affect DNA synthesis (Fig. 5). These findings demonstrate the critical role for MEK/ERK, and possibly JNK, in insulin-inducible endothelial cell proliferation, and the lack of p38 kinase involvement in this process.

Insulin Stimulates Endothelial Cell Regrowth After Mechanical Injury In Vitro in an Egr-1-Dependent Manner

Mechanically wounding vascular endothelial (and smooth muscle) cells in culture results in migration and proliferation at the wound edge and the eventual recovery of the denuded area. We hypothesized that insulin would accelerate this cellular response to mechanical injury. Acutely scraping the growth-quiescent (rendered by 48 h incubation in 0.25% serum) endothelial monolayer resulted in a distinct wound edge (Fig. 6A). Continued incubation of the cultures in a medium containing low serum for a further 3 days resulted in weak regrowth in the denuded zone (Fig. 6B) but aggressive regrowth in the presence of optimal amounts of serum (10%) (Fig. 6C). When insulin (500 nM) was added to growth-quiescent cultures at the time of injury (Fig. 6D) the population of cells in the denuded zone significantly increased, albeit as expected, less efficiently than the 10% serum control (compare Fig. 6B, D).

To investigate the involvement of Egr-1 in endothelial regrowth potentiated by insulin after injury we incubated the cultures with antisense Egr-1 oligonucleotides prior to scraping and again at the time of injury and the addition of insulin. AS2 (0.8 μM) significantly inhibited endothelial regrowth stimulated by insulin (Fig. 6E). In contrast, regrowth in the presence of AS2C (0.8 μM) was not significantly different from cultures in which oligonucleotide was omitted (Fig. 6F). Similar findings were observed when higher concentrations (1.2 μM) of AS2, and AS2C were used (compare Fig. 6H, G). Thus, endothelial regrowth after injury is stimulated by insulin and proceeds in an Egr-1-dependent manner. These observations, quantitated in Fig. 6I, clearly demonstrate that insulin stimulates endothelial regrowth following injury.

Insulin Potentiates Smooth Muscle Cell Regrowth in Response to Mechanical Injury

Finally, we examined the effect of insulin on the reparative response of smooth muscle cells to injury since smooth muscle cells are involved in the pathogenesis of atherosclerosis and restenosis. Increased smooth muscle repair in the presence of insulin may account for the higher increased incidence of early restenosis in diabetics [Stein et al., 1995]. We hypothesized, based on our preceding findings, that Egr-1 may also be involved in the reparative response of smooth muscle cells. Western blot analysis revealed that levels of Egr-1 protein in these cells exposed to insulin were attenuated by AS2, whereas AS2C had no effect (Fig. 7A). The effect of these oligonucleotides on the capacity of insulin to augment smooth muscle cell repair was determined after injury in vitro. AS2 (0.8 μM) inhibited smooth muscle cell regrowth potentiated by insulin (Fig. 7B) while AS2C had little effect (Fig. 7B), thus providing the first demonstration that Egr-1 plays a neces-

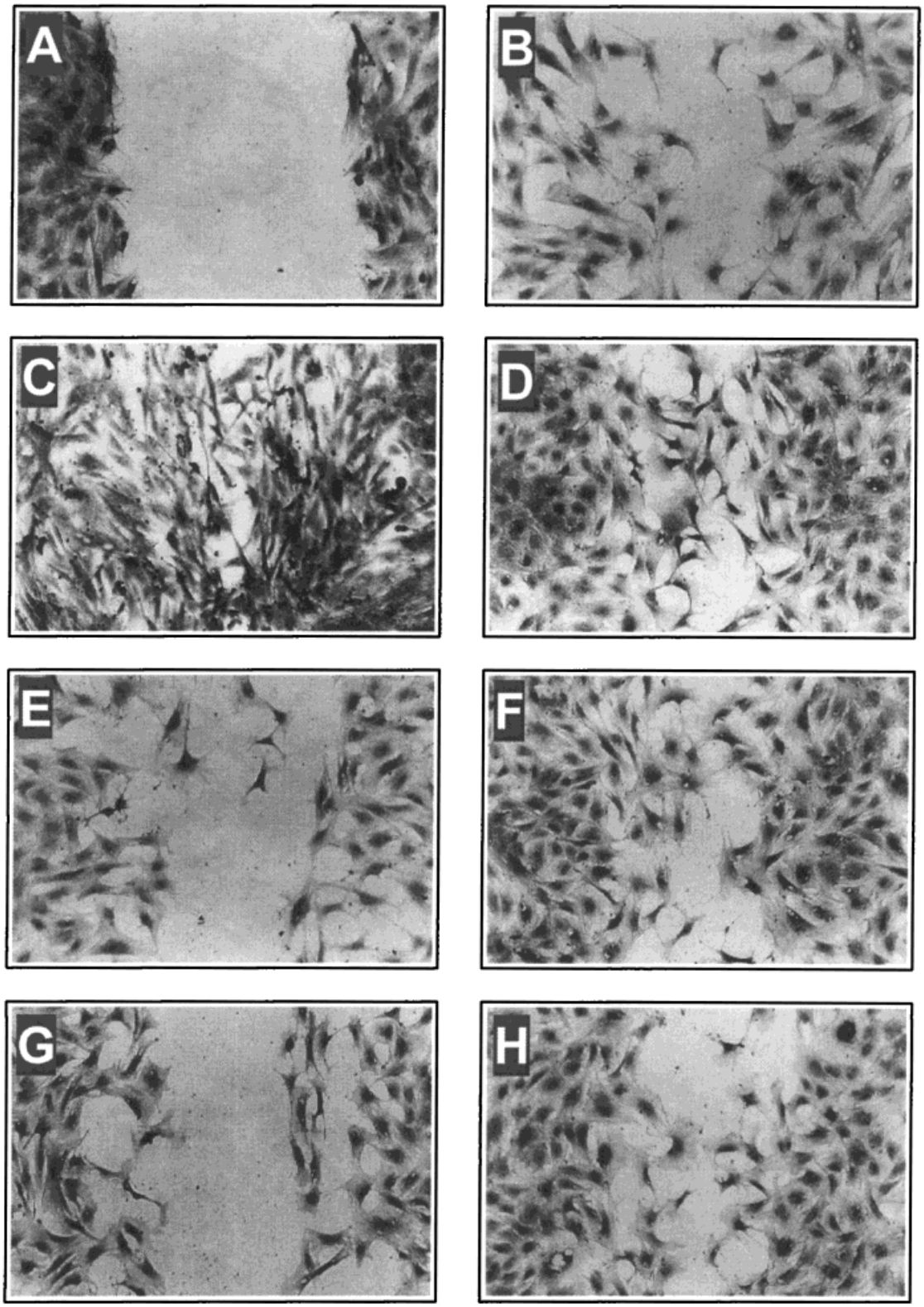

Fig. 6. Wound repair after endothelial injury is potentiated by insulin in an Egr-1-dependent manner. Growth-quiescent endothelial cells were (**A**) scraped with a sterile toothpick and (**B**) left undisturbed for 3 days or incubated with (**C**) 10% FBS or (**D**) 500 nM insulin at the time of injury then left for 3 days. Alternatively, the endothelial monolayers were incubated with 0.8 μM of (**E**) AS2 or (**F**) AS2C, or 1.2 μM (**G**) AS2 or (**H**) AS2C, prior to injury. Cultures were stained with hematoxylin/eosin and photographed. Representative photomicrographs are shown. The population of cells in the denuded zone 3 days after injury in the various groups was quantitated and presented histodiagrammatically in (**I**).

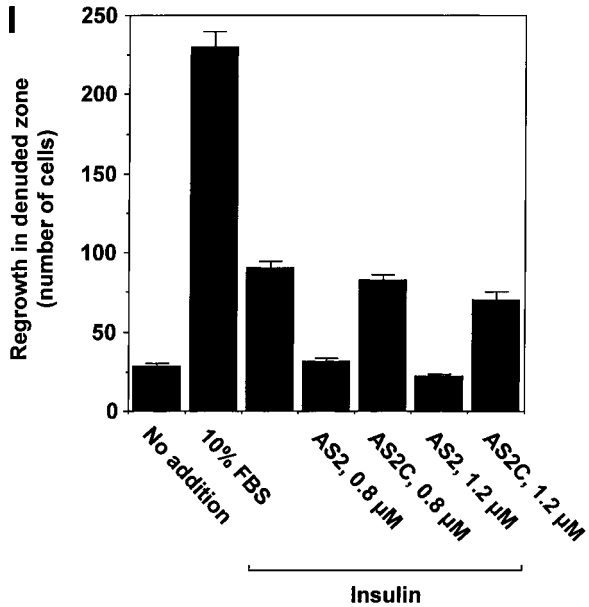

Fig. 6. (*Continued*)

sary role in insulin-assisted smooth muscle cell growth. Taken together, these findings demonstrate the functional importance of Egr-1 in two vascular cell types exposed to insulin.

DISCUSSION

In this paper, we have demonstrated that insulin-induced proliferation and regrowth after injury are processes critically dependent upon the activation of Egr-1. Northern blot, RT-PCR, and Western immunoblot analysis reveal that insulin induces Egr-1 mRNA and protein expression. Antisense oligonucleotides which block insulin-induced synthesis of Egr-1 protein in a sequence-specific and dose-dependent manner, also inhibit proliferation and regrowth after mechanical injury. These findings using nucleic acids specifically targeting Egr-1 demonstrate the functional involvement of this transcription factor in insulin-inducible signaling in two distinct primary cell types.

The capacity of insulin to stimulate proliferation in endothelial cells is controversial. For example, insulin stimulates DNA synthesis in bovine corneal [Feldman et al., 1993; Crow et al., 1994] and retinal [King et al., 1983] endothelium, but has no significant stimulatory effect on endothelium derived from guinea pig coronary [Schonwald et al., 1991], human umbilical vein [Taggart and Stout, 1980; King et al., 1983] and bovine aortic [King et al., 1983]

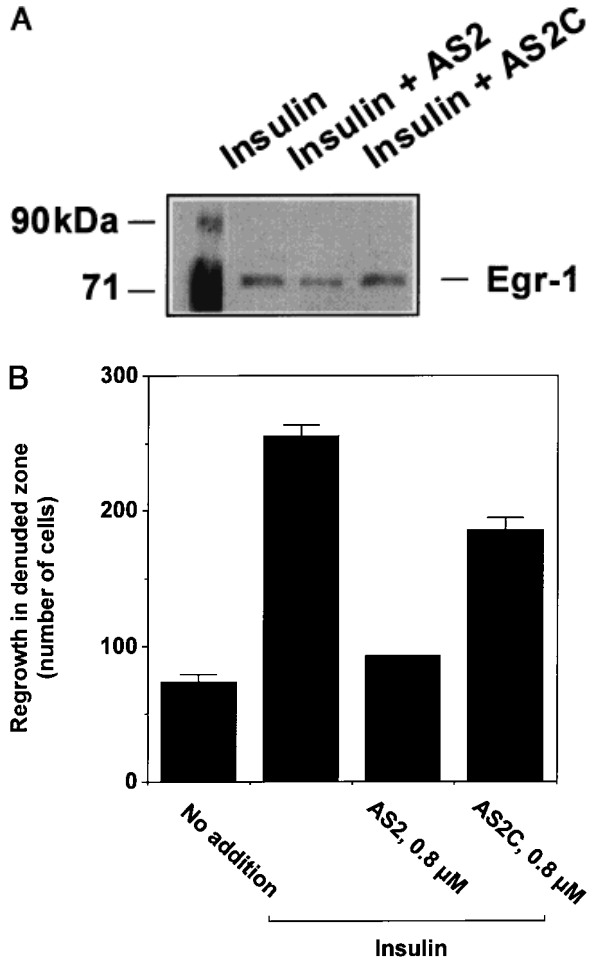

Fig. 7. Wound repair after smooth muscle cell injury is stimulated by insulin in an Egr-1-dependent manner. Growth-arrested smooth muscle cells were incubated with 0.8 μM of AS2 or AS2C, where indicated, prior to (**A**) stimulation with serum (10% FCS) for 1 h and Western blot analysis with antibodies to Egr-1, or (**B**) in vitro injury. Cultures in (B) were stained after 3 days, photographed, and the number of cells residing in the denuded zone enumerated.

endothelial cells. King et al. used Scatchard analysis to demonstrate capacity of ^{125}I-insulin to bind high-affinity receptors on bovine retinal capillary endothelial and aortic endothelial cells [King et al., 1983], and suggested differences in the responsiveness of endothelial cells derived from capillaries and large vessels to insulin. In this study we found that insulin was an effective mitogen to bovine aortic endothelial cells. The reasons for the apparent discrepancy between the previous findings and the present data are not clear but may be related to the preparation of insulin, extent of growth arrest prior to insulin exposure and passage number of the cells. The capacity of

insulin to stimulate proliferation in vascular smooth muscle cells is well established [King et al., 1983; Xi et al., 1997; Begum et al., 1998].

Insulin signaling involves the activation of a growing number of immediate-early genes and transcription factors. These include c-*fos* [Mohn et al., 1990; Jhun et al., 1995; Harada et al., 1996], c-*jun* [Mohn et al., 1990], nuclear factor-B [Bertrand et al., 1998], SOCS3 [Emanuelli et al., 2000] and the forkhead transcription factor FKHR [Nakae et al., 1999]. Insulin also induces the expression of Egr-1 in mesangial cells [Solow et al., 1999], fibroblasts [Jhun et al., 1995], adipocytes [Alexander-Bridges et al., 1992] and Chinese hamster ovary cells [Harada et al., 1996]. This study to the best of our knowledge, is the first to describe the induction of Egr-1 by insulin in vascular endothelial cells (Figs. 2, 3) or smooth muscle cells (data not shown). It also demonstrates a necessary and sufficient role for Egr-1 in the reparative response of both cell types to mechanical injury in the presence of insulin.

Insulin activates several subclasses within the MAP kinase superfamily, including ERK, JNK, and p38 kinase [Guo et al., 1998]. Our findings indicate that the specific ERK inhibitor PD98059, which binds to MEK and prevents phosphorylation by Raf, inhibits insulin-inducible endothelial cell proliferation. Egr-1 transcription is itself dependent upon the phosphorylation activity of ERK via its activation of ternary complex factors (such as Elk-1) at serum response elements (SRE) in the Egr-1 promoter. Six SREs appear in the Egr-1 promoter whereas only one is present in the c-*fos* promoter [Gashler and Sukhatme, 1995]. PD98059 blocks insulin-inducible Elk-1 transcriptional activity at the c-*fos* SRE in vascular cells [Xi et al., 1997]. These published findings are consistent with our demonstration of the involvement of Egr-1 in insulin-inducible proliferation.

Endothelial dysfunction is considered to be an early event in cardiovascular complications associated with diabetes [Sank et al., 1994; McLaren et al., 1999]. Endothelial cells derived from the umblical cords of insulin-dependent diabetic mothers demonstrate two- to eightfold greater proliferation compared to normals [Sank et al., 1994]. Endothelial cell hyperplasia is associated with vascular hypertrophy in diabetic rats [Vranes et al., 1999]. Activation of normally quiescent endothelium could lead to platelet and monocyte adhesion, increased lipid accumulation in the subintima, contraction, and migration and proliferation of underlying smooth muscle cells. Aggressive smooth muscle cell growth after mechanical injury is a central feature in post-angioplasty restenosis, whose incidence is significantly increased in diabetes [Stein et al., 1995; Kip et al., 1996]. Many of the mediators that positively influence smooth muscle and endothelial growth are governed by the transcriptional activity of Egr-1 [Khachigian and Collins, 1998]. Accordingly, we also investigated herein the effect of Egr-1 blockade on smooth muscle cell regrowth after injury. Our demonstration in vitro that Egr-1 plays a critical role in the process of insulin-assisted smooth muscle repair suggest that this transcription factor may be an important target for therapeutic strategies in the future clinical management of diabetes.

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