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Antiproliferative Effect of Elevated Glucose in Human Microvascular Endothelial Cells

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Abstract Diabetic microangiopathy has been implicated as a fundamental feature of the pathological complications of diabetes including retinopathy, neuropathy, and diabetic foot ulceration. However, previous studies devoted to examining the deleterious effects of elevated glucose on the endothelium have been performed largely in primary cultured cells of macrovessel origin. Difficulty in the harvesting and maintenance of microvascular endothelial cells in culture have hindered the study of this relevant population. Therefore, the objective of this study was to characterize the effect of elevated glucose on the proliferation and involved signaling pathways of an *immortalized* human dermal microvascular endothelial cell line (HMEC-1) that possess similar characteristics to their in vivo counterparts. Human dermal microvascular endothelial cells (HMEC-1) were grown in the presence of normal (5 mM) or high D-glucose (20 mM) for 14 days. The proliferative response of HMEC-1 was compared under these conditions as well as the cAMP and PKC pathways by in vitro assays. Elevated glucose significantly inhibited ($P < 0.05$) HMEC-1 proliferation after 7, 10, and 14 days. This effect was not mimicked by 20 mM mannitol. The antiproliferative effect was more pronounced with longer exposure (1–14 days) to elevated glucose and was irreversible 4 days after a 10-day exposure. The antiproliferative effect was partially reversed in the presence of a PKA inhibitor, Rp-cAMP (10–50 μM), and/or a PKC inhibitor, Calphostin C (10 nM). HMEC-1 exposed to elevated glucose (20 mM) for 14 days caused an increase in cyclic AMP accumulation, PKA, and PKC activity but was not associated with the activation of downstream events such as CRE and AP-1 binding activity. These data support the hypothesis that HMEC-1 is a suitable model to study the deleterious effects of elevated glucose on microvascular endothelial cells. Continued studies with HMEC-1 may prove advantageous in delineation of the molecular pathophysiology associated with diabetic microangiopathy. J. Cell. Biochem. 71:491–501, 1998.

Key words: diabetic microangiopathy; endothelium; HMEC-1

Hyperglycemia is a key metabolic abnormality in diabetes mellitus that is believed to play a significant role in the development of retinopathy, nephropathy, and neuropathy [Kamal, 1996; Nathan, 1993]. Diabetic microangiopathy causes end organ damage that involves different vascular beds including retina, kidney, nerves, and diabetic foot pathology [Kamal, 1996].

Previous studies have shown suppression of endothelial cell proliferation after treatment with elevated glucose using human umbilical vein endothelial cells (HUVECs) or bovine carotid endothelial cells [Stout, 1982; Lorenzi, 1985, 1987; Hayashi, 1991]. For example, Lorenzi et al. [1985] showed a delay of saturation density of HUVECs in the presence of elevated glucose concentration (20 mM) and a more profound reduction in cell number at 40 mM glucose. Likewise, elevated glucose decreases thymidine uptake in microvascular endothelial cells derived from retina [Naeser, 1986] and cardiac capillaries of diabetic rats [Wickline, 1985]. However, increased thymidine incorporation has been shown in retinal endothelial cells in diabetic animal models [Cuthbertson, 1989; Sharma, 1985].

Recently, great attention has been focused to unravel the underlying mechanisms of chronic complications of diabetes mellitus. Chronically elevated glucose is shown to cause stimulation of the polyol pathway, activation of the diacylglyerol-protein kinase pathway, formation of free

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radicals, and the formation of advanced glycosylated end products [Kamal, 1996]. Substantial evidence exists that elevated glucose activates the protein kinase C (PKC) signaling pathway both in vitro [Lee, 1989] and in various damage prone tissues of diabetic animals [Craven, 1989; Shiba, 1993]. Functional studies with PKC inhibitors have been shown to prevent the glucose-induced vascular abnormalities in granulation tissue [Wolf, 1991] aberrant contractility in rabbit aorta [Tesfamariam, 1991], and restore the vascular dysfunctions in diabetic rats [Ishii, 1996].

A role of the PKA signaling pathway in the mediation of the deleterious effects of elevated glucose is suggested by the observation of elevated cAMP levels in renal glomeruli of diabetic animals [Thomas, 1992]. In animal studies, diabetes is shown to produce alteration in adenylate cyclase activity of cerebral microvascular endothelial cells [Palmer, 1983] and cAMP phophodiesterase activity in adipose tissue [Solomon, 1990]. Interestingly, cyclic AMP has also been shown to modulate dermal microvascular endothelial proliferation and phenotype in vitro [Davison, 1981; Tuder, 1990].

The objective of these studies was to characterize the antiproliferative effect of high glucose and to delineate the possible role of the cAMP and PKC pathways in mediating the effect in HMEC-1. HMEC-1 were chosen for this study based on their less stringent growth requirements and potential for longer term study as compared to normal human microvascular endothelial cells [Ades, 1992; Pruckler, 1993; Xu, 1994]. These cells provide a reliable source of human microvascular endothelial cells devoid of mycoplasma and viral infection [Xu, 1994]. HMEC-1 are the first immortalized microvascular cell line that retain the morphological, phenotypic, and functional characteristics of their in vivo counterparts [Ades, 1992; Pruckler, 1993; Xu, 1994]. These properties include a typical cobblestone morphology, expression and secretion of von Willebrand's Factor, uptake of aceylated LDL, tube-formation when cultured on Matrigel, expression of CD31 and CD36 cell surface molecules, expression of ICAM-1 and CD44 cell adhesion molecules, and specific binding of lymphocytes in cell adhesion assays [Ades, 1992]. HMEC-1 have been successfully used as a model system to evaluate cholesterol uptake [Xu, 1994]. To our knowledge, this represents the first study devoted to examining the response of HMEC-1 to elevated glucose.

MATERIALS AND METHODS

Immortalized human dermal microvascular endothelial cell line (HMEC-1) were obtained from Dr Edwin W. Ades (Biological Product Branch, Center for Disease Control and Prevention, Atlanta, GA) and Dr Thomas J. Lawley (Emory University, Atlanta, GA). HMEC-1 were recently established by transfection of human dermal microvascular endothelial cell with a PBR-322-base plasmid containing the coding region for simian virus 40 A gene product, large T antigen [Ades, 1992; Pruckler, 1993; Xu, 1994].

HMEC-1 were grown in medium MCDB-131 (Sigma, St. Louis, MO) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 10 ng/ml EGF (Collaborative Biochemical Products, Bedford, MA), 1 µg/ml hydrocortisone (Sigma), 100 U/ml penicillin-streptomycin, and 5 U/ml Fungizone (Gibco BRL, Grand Island, NY) at 37°C in a humidified 5% CO_2 incubator. Glucose concentration in control medium was 5 mM. Elevated glucose was studied at a concentration of 20 mM.

HMEC-1 Proliferation Studies

HMEC-1 were seeded onto six-well polystyrene plates. After 24 h (Day 0), medium was changed to 5 mM D-glucose, 20 mM D-glucose, or 20 mM mannitol. HMEC-1 proliferation was determined on days 0, 3, 7, 10, and 14. HMEC-1 proliferation was determined by Coulter Counter (Coulter Electronics Inc., Hialeah, FL) analysis of a trypsinized aliquot of cells. We have utilized a periodic cell count method since previous thymidine incorporation studies have yielded conflicting data [Nasser, 1986; Wickline, 1985; Cuthbertson, 1989; Sharma, 1985], are limited to detect S phase perturbations, and are fraught with technical pitfalls. These include extranuclear binding of thymidine, dependence upon cellular uptake and metabolism of thymidine in the cell, and problems with the physicochemical properties of [^{3}H] thymidine [Maurer, 1981]. Studies were repeated in the presence of Rp-cAMP (10–50 µM) and/or Calphostin C (10nM) to inhibit PKA and PKC, respectively. Rp-cAMP is a diasteromer of cAMP that competitively binds to the regulatory subunit of PKA to prevent cAMP-induced dissociation and activation of the enzyme [Rothermel,

1983]. Calphostin C inhibits PKC activity by binding to the regulatory domain of PKC [Kobayashi, 1989] and was preactivated by a 3 h exposure to light [Bruns, 1991]. Both inhibitors were present throughout the entire duration of glucose exposure including fresh administration along with medium changes every 48 h.

Assay of cAMP Production

After 14 days exposure to 5 mM D-glucose and 20 mM D-glucose, HMEC-1 were treated with 10 mM 3-isobutyl, 1-methylxanthine (IBMX) for 1 h. Medium was aspirated and ice-cold 95% (v/v) n-propanol was added for 20 min. Extracts were collected and dried in a Savant Speedvac (Farmingdale, NY). Dried extracts were resuspended in a buffer containing 0.05 mM sodium acetate (pH 6.2) and 0.01% sodium azide. Cyclic AMP was assayed in the dried extracts using a commercially available RIA kit (Biomedical Technologies Inc., Stoughton, MA) as described [Iba, 1992].

Measurement of Protein Kinase C and Protein Kinase A Activity

Following 14 days exposure to 5 mM and 20 mM D-glucose, HMEC-1 were washed with cold PBS and harvested mechanically in cold PBS using a rubber policeman. The HMEC-1 suspension was then centrifuged at 300*g* at 4°C for 5 min. The HMEC-1 pellet was resuspended in harvest buffer containing 20 mM Tris (pH 7.4), 1 mM EGTA, 1 mM DTT, 0.5 mg leupeptin and 25 kallikrein units of aprotinin. HMEC-1 were homogenized with ten strokes using a Dounce (type A) homogenizer. Protein kinase C and protein kinase A was measured by its ability to phosphorylate the substrate Pep Tag C1 peptide or Pep Tag A1 peptide (Kemptide) using Pep Tag™ nonradioactive protein kinase C or cAMP dependent protein kinase assay kit (Promega, Madison, WI).

Nuclear Extract Preparation and Gel Shift Assay

HMEC-1 were exposed to 5 mM D-glucose or 20 mM D-glucose for 14 days. Nuclear extracts were isolated as described in detail previously [Digman, 1983; Du, 1995]. Gel shift assay was performed using AP-1 or CRE DNA Binding Protein Assay System (Gibco BRL, Gaithersburg, MD). The oligonucleotide, containing the tandem repeat of the consensus sequence for the AP-1 binding site (5'-GATCCTTCGTGAGTCAGCGGGAT-CCTTCGTGAGTCAGCGG-3') or CRE binding site (5'-GATCTGACGTCATGAC-TGA-CGTCATGACTGACGTCATCA-3') were labeled with [γ^{-32} P] ATP by T4 kinase. Nuclear extracts were then incubated with the radioactive oligonucleotide at room temperature for 15 min. The mixture was separated by electrophoresis on a 6% (w/v) nondenaturing polyacrylamide gel in 0.25 × TBE. The dried gel was exposed to X-ray film. For testing the specificity of the gel shift assay, unlabeled oligonucleotide was used as a competitor. Densitometry was performed with a Visage 2000 densitometer (BioImage, Ann Arbor, MI).

Statistical Analysis

Data are expressed as mean ± SEM and were analyzed by one-way analysis of variance and a Kruskal-Wallis post-test or by unpaired *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Effect of Elevated D-Glucose Concentrations on HMEC-1 Proliferation

To determine the effect of elevated ambient glucose on HMEC-1 proliferation, HMEC-1 were grown in the presence of 5 mM D-glucose, 20 mM D-glucose, or 40 mM D-glucose for 14 days. HMEC-1 proliferation in the presence of 20 mM D-glucose was significantly ($P < 0.01$) less than that observed at 5 mM D-glucose at days 10 and 14 (Fig. 1). In some experiments, the antiproliferative effect of elevated glucose was apparent by day 7 (data not shown). HMEC-1 grown in 40 mM D-glucose showed more pronounced inhibition ($P < 0.01$) on day 10 and day 14 as compared to HMEC-1 in 5 mM or 20 mM D-glucose. In addition, the ability of elevated glucose to inhibit HMEC-1 proliferation was not attributable to hyperosmolality since it was not mimicked by 20 mM mannitol (data not shown).

Reversibility of Glucose Inhibition on HMEC-1 Proliferation

In order to test whether the antiproliferative effect of elevated glucose on HMEC-1 was reversible, HMEC-1 were exposed to 5 mM and 20 mM D-glucose for 14 days. In the assigned group of HMEC-1 maintained at high glucose, medium was changed to 5 mM D-glucose on day 10. HMEC-1 grown in 20 mM D-glucose showed significant inhibition of proliferation ($P < 0.01$) as compared to 5 mM D-glucose on

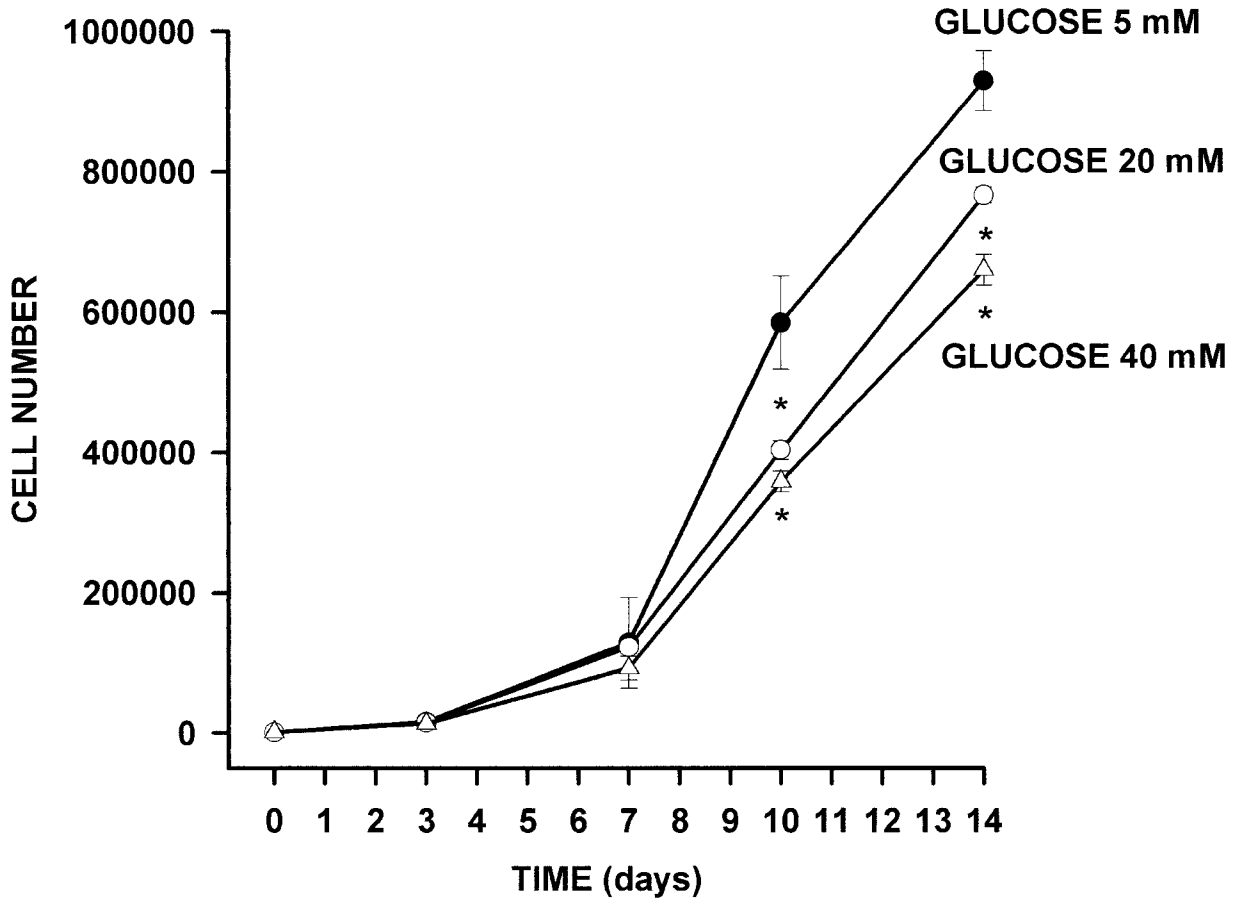

Fig. 1. Effect of elevated glucose on HMEC-1 proliferation. HMEC-1 were incubated with the indicated concentrations of D-glucose for up to 14 days. Initial seeding density at day 1 was 1,000 cells/well with 1,171 ± 139 cells at day 0. Data shown are from a representative experiment repeated three times. *$P < 0.01$ vs. glucose (5 mM).

days 10, 14, and 17. The substitution of medium after 10 days from elevated to normal glucose failed to prevent or reverse the antiproliferative effect of glucose on day 14 (Fig. 2) or day 17 (data not shown).

Effect of Protein Kinase A or Protein Kinase C Inhibitor on HMEC-1 Proliferation

HMEC-1 proliferation was studied in the absence or presence of a PKA inhibitor, 10 µM Rp-cAMP (Rp; a diasteromer of adenosine cyclic 3', 5' phosphorothioate), a PKC inhibitor, 10 nM Calphostin C, and a combination of both inhibitors. As shown in Figure 3A, Rp-cAMP (10 µM) did not affect HMEC-1 proliferation in the control group (5 mM glucose). However, Rp-cAMP (10 µM) partially reversed the antiproliferative effect of high glucose by approximately 20% ($P < 0.05$; Fig. 3A). Treatment with a higher concentration of Rp-cAMP (50 µM) failed to reverse the antiproliferative effect of glucose to a greater degree than that observed in the presence of 10 µM Rp-cAMP (data not shown). The antiproliferative effect of high glucose was slightly lessened ($P < 0.05$) in the presence of Calphostin C but this may reflect the antiproliferative action found by Calphostin C alone in the control group (5 mM glucose; Fig. 3B). Treatment with Rp-cAMP (50 µM) and Calphostin C (10 nM) failed to provide any additive effect as compared to that observed in the presence of the inhibitors independently (Fig. 3C).

Cyclic AMP Production/Protein Kinase A Activity (PKA)/Binding of CRE Binding Protein

Cyclic AMP accumulation was determined in HMEC-1 after 14 days of 5 mM D-glucose or 20 mM D-glucose exposure. Cyclic AMP production in HMEC-1 exposed to 20 mM D-glucose for 14 days was significantly ($P < 0.05$) increased in six of seven experiments (Fig. 4A). After a 14 day exposure to 20 mM D-glucose, HMEC-1 showed a 20% increase ($P < 0.005$) in the activity of PKA (Fig. 4B). However, CRE

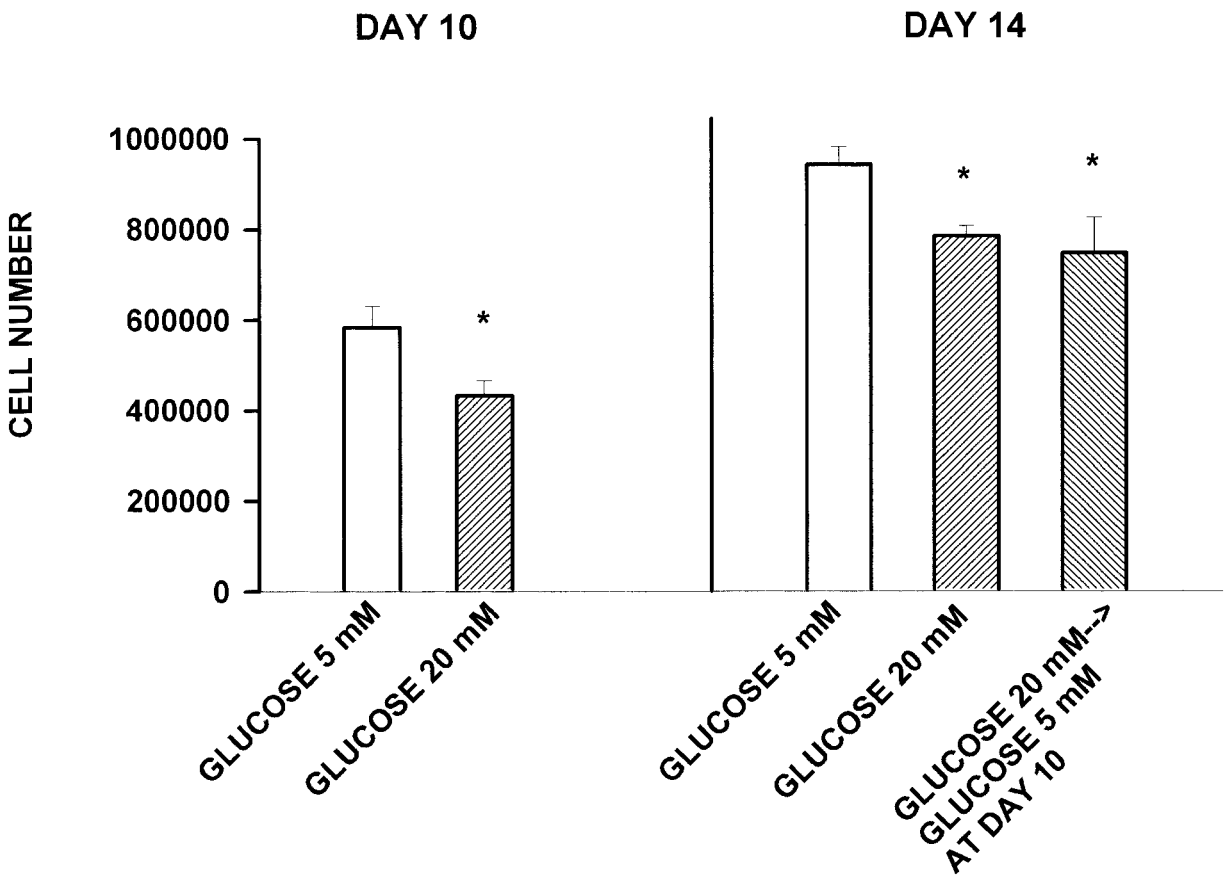

Fig. 2. Determination of reversibility of glucose-mediated antiproliferation in HMEC-1. HMEC-1 were exposed to elevated (20 mM) or normal (5 mM) glucose for 10 days (left side of figure). After 10 days, HMEC-1 were maintained in high (20 mM), normal glucose (5 mM), or switched from high to normal glucose. HMEC-1 proliferation was then measured at Day 14 (right side of figure). $*P < 0.01$ vs. glucose (5 mM). Data shown are from a representative experiment repeated three times.

binding showed no significant increase in HMEC-1 treated with high glucose (20 mM) as compared to normal glucose (5 mM) for 14 days (Fig. 4C).

Protein Kinase C (PKC) and AP-1 Binding

A 14 day exposure to 20 mM D-glucose raised the PKC activity by 40% as compared to HMEC-1 grown in the presence of 5 mM D-glucose (Fig. 5A). AP-1 binding activity of HMEC-1 was enhanced in some experiments after a 14 day incubation period in the presence of 20 mM glucose as shown in the Figure 5B inset. However, densitometric analysis of five such experiments failed to show a significant elevation of AP-1 binding (Fig. 5B).

DISCUSSION

Diabetes mellitus is known to cause diverse complications as a result of tissue damage in various organs. Among other causes of tissue damage, diabetic microangiopathy stand out to be the most consistent pathological finding in diabetic nephropathy, neuropathy, and retinopathy [Kamal, 1996]. Recent trials have established hyperglycemia as major risk factor in the development of diabetic microangiopathy [Skyler, 1996]. To understand the interaction between elevated glucose and various aspects of microangiopathy, several studies have investigated endothelial proliferation or DNA synthesis in high glucose. However, these studies have been conducted predominantly in endothelial cells derived from macrovessel origin. We sought to test whether similar findings would be obtained in an immortalized human dermal microvessel endothelial cell line. If correct, the longevity and ease of maintenance of HMEC-1 cells would be ideally suited for the delineation of the deleterious effects of chronic elevated

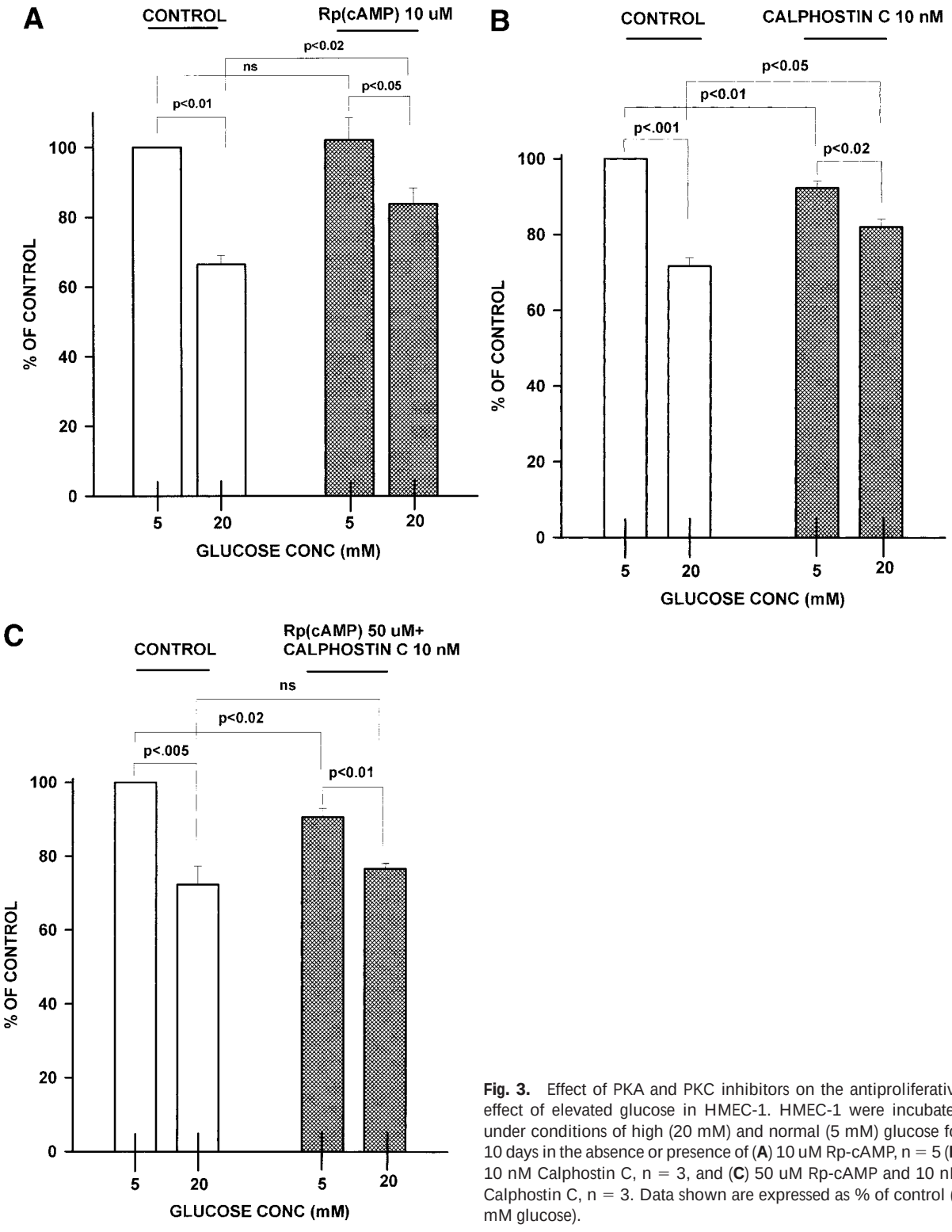

Fig. 3. Effect of PKA and PKC inhibitors on the antiproliferative effect of elevated glucose in HMEC-1. HMEC-1 were incubated under conditions of high (20 mM) and normal (5 mM) glucose for 10 days in the absence or presence of (**A**) 10 uM Rp-cAMP, n = 5 (**B**) 10 nM Calphostin C, n = 3, and (**C**) 50 uM Rp-cAMP and 10 nM Calphostin C, n = 3. Data shown are expressed as % of control (5 mM glucose).

glucose as found in the microvasculature of diabetic subjects.

Our data shows that elevated glucose (20 mM or 40 mM) significantly inhibits the proliferation of microvessel endothelial cells compared to 5 mM D-glucose. We also observed concentration-dependent inhibition of HMEC-1 proliferation when 20 mM and 40 mM D-glucose concentrations were compared. This is consistent with a previous study that utilized a large vessel endothelial cell proliferation assay to demonstrate concentration-dependent antiprolifera-

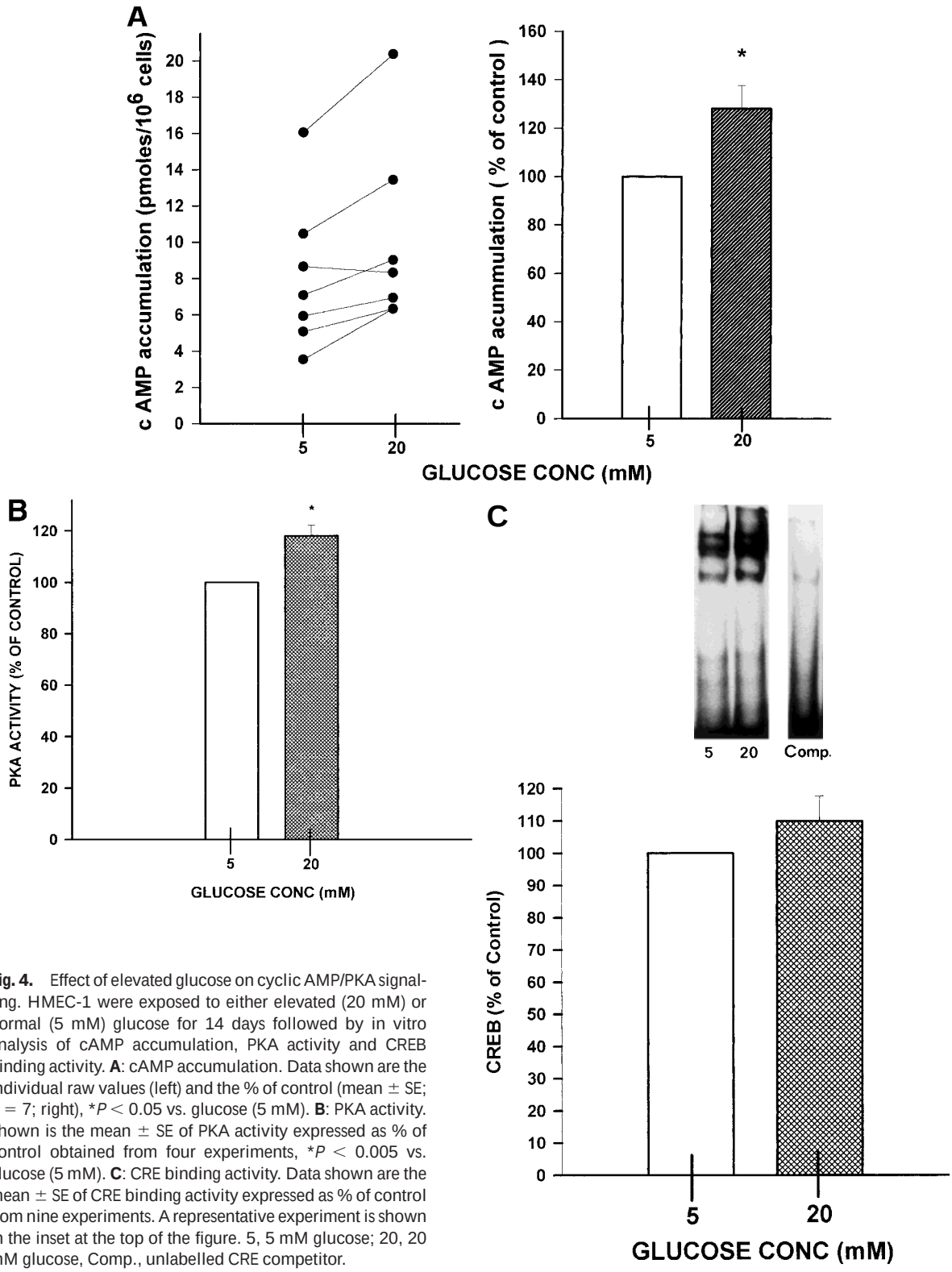

Fig. 4. Effect of elevated glucose on cyclic AMP/PKA signalling. HMEC-1 were exposed to either elevated (20 mM) or normal (5 mM) glucose for 14 days followed by in vitro analysis of cAMP accumulation, PKA activity and CREB binding activity. **A**: cAMP accumulation. Data shown are the individual raw values (left) and the % of control (mean ± SE; n = 7; right), $*P < 0.05$ vs. glucose (5 mM). **B**: PKA activity. Shown is the mean ± SE of PKA activity expressed as % of control obtained from four experiments, $*P < 0.005$ vs. glucose (5 mM). **C**: CRE binding activity. Data shown are the mean ± SE of CRE binding activity expressed as % of control from nine experiments. A representative experiment is shown in the inset at the top of the figure. 5, 5 mM glucose; 20, 20 mM glucose, Comp., unlabelled CRE competitor.

tive effects of elevated glucose in HUVEC [Lorenzi, 1985]. Similarly, our data confirm earlier findings obtained with HUVEC demonstrating a failure of mannitol to mimic the antiproliferative effect of glucose [Lorenzi, 1985].

In initial studies, Lorenzi et al. [1987] reported a glucose-induced marginal delay in endothelial cell (HUVEC) transit through S and G_2 phases but the opposite was shown by Curcio et al. [1992] in similar studies. Our cell cycle

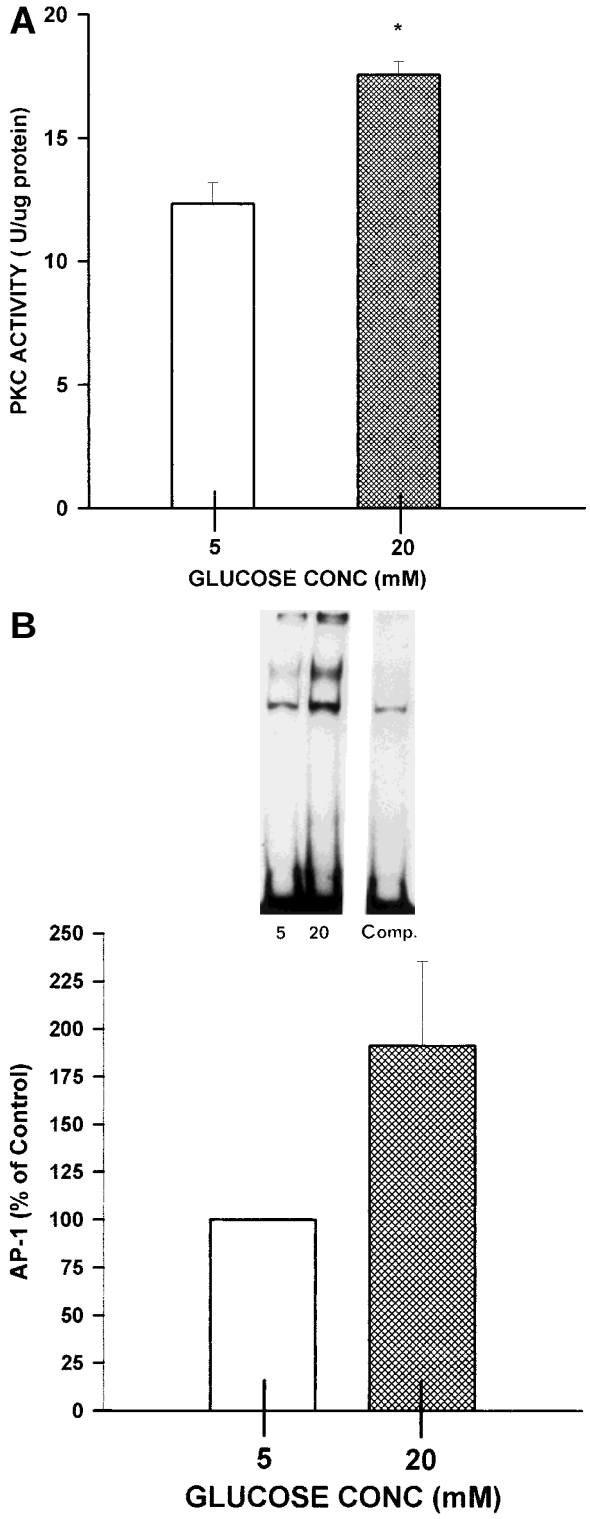

Fig. 5. Effect of elevated glucose on PKC/AP-1 binding activities. HMEC-1 were exposed to either elevated (20 mM) or normal (5 mM) glucose for 14 days followed by in vitro analysis of PKC and AP-1 binding activities. **A**: PKC activity. Data shown are the mean ± SE from four experiments. *$P < 0.005$ vs. glucose (5 mM). **B**: AP-1 binding activity. Data shown are the mean ± SE of AP-1 binding activity expressed as % of control from five experiments. A representative experiment is shown in the inset at the top of the figure. 5, 5 mM glucose; 20, 20 mM glucose, Comp., unlabelled AP-1 competitor.

studies demonstrate slight but insignificantly increased proportion of HMEC-1 in G_0-G_1 and identical numbers of HMEC-1 in S and G_2 phases in cells exposed to elevated glucose as compared to normal glucose (data not shown). The variable results obtained from our work and other studies may reflect inherent limitation of different methods and cell lines used in cell cycle studies.

The ability of elevated glucose to inhibit HMEC-1 proliferation required a lag phase of 24 to 72 h for its maximal effect to be achieved (data not shown). Another characteristic of the antiproliferative effect of glucose is that once fully established, it is nearly irreversible, such that the growth of HMEC-1 cells upon restoration of glucose concentrations from high (20 mM) to normal (5 mM) concentrations was not different from that observed in cells maintained at high concentrations. Several reports have observed lag periods of variable duration both in cell proliferation studies [Lorenzi, 1985] and accumulation of extracellular matrix proteins after glucose exposure [Ayo, 1990]. Despite a paucity of direct evidence, incomplete reversibility of the antiproliferative effect of elevated glucose has been attributed to DNA damage to form increased single-strands breaks as suggested by [Lorenzi, 1986] and persistence of defective gene expression through several cell divisions despite exposure to normal glucose [Roy, 1990]. Restoration to normal glucose may not be sufficient to reverse or prevent further damage. In this respect, Curcio and Ceriello [1992] recently demonstrated reversal of the antiproliferative of elevated glucose in HUVEC in the presence of different antioxidants. However, the exact mechanism involved remains to be established.

Investigators have speculated as to diverse mechanisms to explain the microangiopathy that results from chronic elevation of glucose [King, 1994]. Among the proposed mechanisms, the DAG-PKC pathway has become the focus of recent interest. Elevated glucose is shown to increase the synthesis of DAG that in turn activates PKC [Lee, 1989]. Inoguchi et al. [1992] showed preferential stimulation of the PKC isoform β2 in the heart and aorta of diabetic rats. Our results also demonstrate elevation of PKC activity in HMEC-1 after 14 days of elevated glucose that was not replicated by shorter exposure of 24 h. Although not examined in the present study, it will be worthwhile to investigate whether elevated glucose stimulates simi-

lar isoforms in HMEC-1. The utility of HMEC-1 as a vehicle for stable transfectants may prove beneficial in delineating gene regulation [Xu, 1994].

cAMP has been shown to be more important for microvascular endothelial proliferation compared to macrovascular endothelium [Davison, 1981]. However, much less is known regarding the cAMP cascade as compared to DAG-PKC signaling in microvascular endothelium exposed to elevated glucose. HMEC-1 demonstrated modest elevation of cAMP levels after exposure to elevated glucose. Cagliero et al. [1991] recently reported no change in cAMP levels in HUVEC when exposed to high glucose [Cagliero, 1991]. We confirm this finding in six of seven experiments with HMEC-1 cells exposed to elevated glucose (Fig. 4A) despite variations in baseline cAMP levels of unknown etiology.

Inhibition of PKA and PKC with specific inhibitors partially reversed the antiproliferative response to elevated glucose in HMEC-1. Inhibition of PKA with 10 μM Rp (cAMP) led to a lessening of the antiproliferative effect of elevated glucose. Utilization of higher concentrations of Rp (50 μM) failed to provide any greater blockade. The lack of stimulation of CRE binding activity with elevated glucose in HMEC-1 suggest that either the PKA pathway may play a minor role in mediating the antiproliferative effect of glucose or that other competing signaling pathways are involved. Failure of PKC inhibitors to produce a more dramatic reversal of the antiproliferative effects of elevated glucose may be a consequence of the broad potency of Calphostin C to inhibit PKC activity by binding to the regulatory domain of PKC. As recently shown by Ishii et al. [1996], study of newly-developed specific inhibitors to PKC β isoforms, may prove more fruitful in assessing the importance of PKC in mediating the deleterious effects of high glucose.

Combined inhibition of PKA and PKC pathways not only failed to provide additive effects but actually suppressed the effects of each individual inhibitor. It is difficult to present a precise explanation for the observed combinatorial effects. It is possible that this effect may result from chemical interaction between two inhibitors or due to suppression of other signal transduction pathways. In addition, the inhibition observed with 10 nM calphostin C alone may indicate toxicity although we failed to observe any changes in HMEC-1 adhesion or morphology.

Signal transduction pathways including cAMP and DAG-PKC pathways influence gene transcription through changes in levels or activity of transcription factors [Hill, 1995]. Although several studies have observed changes in transcription factors in renal tissue, little is known for microvascular endothelium. Kreisberg et al. [1994] have demonstrated increase in mRNA levels of *c-fos* and *c-jun* (AP-1) in human mesangial cells after glucose exposure. These findings were confirmed by another study that showed increased mRNA levels of *c-fos* and *c-jun* in glomerular tissue of diabetic rats [Shankland, 1995]. In the present study, gel shift assays failed to show increased AP-1 binding in HMEC-1 after 14 days exposure to elevated glucose.

Cyclic AMP-inducible promoter responsive sites (CRE) have been identified in several genes that are modulated by CRE-binding protein (CREB) [Lalli, 1994]. Since our results showed elevation of cAMP and PKA activity in HMEC-1, it is conceivable to expect increased CREB activity. Conversely, no change was detected on gel shift assay on prolonged glucose exposure. This finding may be explained by the inability of the assay to distinguish between active and inactive forms of CREB and/or complexity of regulation. Our data confirm previous studies that failed to show any change in CREB mobility or abundance in human mesangial cells [Kreisberg, 1994].

In summary, the results of this study show that elevated D-glucose modestly suppresses HMEC-1 proliferation. The antiproliferative effect of high glucose was more pronounced with prolonged exposure with maximal inhibition attained by 7 to 14 days. Failure of mimicry by an equivalent concentration of mannitol suggests a lack of involvement of hyperosmolality. Our finding of stimulated cAMP and PKA/PKC, but not CRE or AP-1 binding activity, in response to elevated glucose is consistent with the partial effect of PKA and PKC inhibitors. These data illustrate complex regulation of HMEC-1 proliferation with elevated glucose in HMEC-1 and suggest the involvement of many cross-talk pathways. Thus, this study suggests that elevated glucose impair microvascular endothelial cell proliferation through multiple signal transduction pathways that eventually engender diabetic vascular disease and culminate in significant morbidity. Moreover, these stud-

ies provide substantial justification for the utilization of HMEC-1 as a suitable microvascular model to examine the chronic effects of elevated glucose as it relates to diabetic microangiopathy.

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