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VASCULAR SMOOTH MUSCLE CELLS EXHIBIT INCREASED GROWTH IN RESPONSE TO ELEVATED GLUCOSE

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Diabetes mellitus is associated with an increased risk of cardiovascular disease. In order to elucidate the association between hyperglycemia and vascular complications, the growth patterns of vascular smooth muscle cells were studied under high glucose conditions. We examined the effect of culturing porcine aortic smooth muscle cells (PVSMC) in high glucose (25 mM, HG) on total cell protein, cell volume, DNA synthesis and cell number. We observed that cells cultured in HG had higher total cell protein content which was associated with increased cell volume as compared to the cells cultured under normoglycemic conditions (5.5 mM glucose, NG). PVSMC cultured in HG also had 1.4 fold increased growth rate and a greater fetal calf serum - induced DNA synthesis rate compared to cells cultured in NG. These observations suggest for the first time that elevated glucose could lead to both hypertrophic and hyperplastic effects in PVSMC. We also examined protein kinase C (PKC) activities as well as the cellular levels of the 12-lipoxygenase product, 12-hydroxyeicosatetraenoic acid (12-HETE) in NG and HG as possible mechanisms for the enhanced growth effects in HG. The results show that PVSMC cultured in HG have increased PKC activity as well as increased levels of 12-HETE. Therefore hyperglycemia may be linked to accelerated vascular disease by increasing smooth muscle cell growth and proliferation.

Diabetes mellitus is associated with the development of several vascular complications as well as a substantially increased prevalence of atherosclerotic disease and cardiovascular mortality (1-3). However, the mechanism(s) for accelerated atherosclerotic disease in diabetes are unclear. Vascular smooth muscle cell growth and proliferation is one key feature for the development of atherosclerosis (4). Although hyperglycemia in diabetes has been suggested to be a contributing factor to complications, no studies have addressed the direct effects of elevated glucose on vascular smooth muscle cell growth and proliferation. In the present studies we have compared the growth of porcine vascular smooth muscle cells (PVSMC)

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cultured under normal (5.5 mM) and elevated glucose (25mM) conditions mimicking normoglycemia and diabetic hyperglycemia respectively. The effects of glucose on cell protein, cell volume, DNA synthesis as well as cell number, were assessed to determine if elevated glucose has hypertrophic and/or hyperplastic effects in VSMC.

Lee et al (5) have postulated that hyperglycemia - induced complications may arise due to increased protein kinase C (PKC) activity arising from elevated diacylglycerol levels (6). In addition, the 12- and 15-lipoxygenase (LO) pathways have been linked to increased cellular proliferation (7,8). We therefore, also measured PKC activity and cell associated levels of the 12-LO product 12-hydroxyeicosatetraenoic acid (12-HETE) in normal and high glucose as one possible mechanism of altered VSMC growth under high glucose conditions.

MATERIALS AND METHODS

Culture of porcine aortic smooth muscle cells. (PVSMC): Aortas were transported rapidly from the local abattoir on ice. The sections were rinsed with DME media containing penicillin (100 U/ml)/streptomycin (100 μg/ml). The endothelial layer was peeled off, small pieces of the underlying smooth muscle layer were cut off, and placed on sterile 35 mm petri dishes. After three washes, sufficient media (DME normal glucose (5.5mM) + 15% fetal calf serum) was added to cover the entire surface and plates incubated at 37°C in a humidified atmosphere. Smooth muscle cells grow out from the explants within 3-4 days and confluency is achieved in about two weeks. Cells were passaged using trypsin-EDTA and used for experiments from passage 2-6. The identity of the SMC in these explant cultures was confirmed by immunofluorescence with anti-smooth muscle cell myosin antibodies and fluorescein isothiocyanate conjugated goat anti rabbit IgG (Sigma, St. Louis, MO). For studies of cells under hyperglycemic conditions, the cells were allowed to grow through at least two passages in DME high glucose (25 mM) before use. Control for osmolality were cells grown for at least two passages in 5.5 mM glucose + 19.5 mM mannose.

Growth Curves: PVSMC that had been through at least two passages in normal (5.5 mM, NG) or high glucose (25 mM, HG) were plated on 35 mm dishes (30,000 cells per dish) in DME/HEPES NG or HG containing 10% fetal calf serum (FCS). Media was changed every 48 hr and cell counts obtained on a Coulter counter. In order to compare growth responses in the absence of serum, cells were plated as above in media containing 10% FCS for 24 hr. Media was then replaced with fresh medium containing 0.2% BSA and 0.3% FCS and cell counts were obtained every 48 hr.

Protein synthesis: Confluent PVSMC in NG or HG in 12 well dishes were serum starved by placing in media + 0.2% BSA + 0.4% FCS for 24 hr. FCS (10%) was added to some wells and cells incubated for 48 hr. Cell monolayers were then washed twice with PBS, lysed with 0.3 ml/well of 0.3N NaOH and total cell protein determined by Lowrys' method (9).

Measurement of Cell Volume: Quiescent PVSMC in NG or HG in 100 mm dishes were treated with a 24 hour pulse of 10% FCS. Cells were trypsinised, resuspended into isotonic saline solution (10 ml) and counted on a Coulter counter (Channelyzer 256). The distribution analyzer connected to the Coulter counter then accumulates the cell counts into histogram form which is then used for quantitation of cell volume from cell diameters. For measurement of cell

volume using Flow cytometry, the experiment was performed as above, stored on ice and the scatter distribution analyzed for cell volume on a Becton Dickinson FACS/4 cell sorter.
DNA synthesis -[^{3}H] Thymidine incorporation. Confluent cells in NG or HG in 12 well dishes were made quiescent for 24 hr in media + 0.2% BSA + 0.4% FCS. Cells were then stimulated with 10% FCS for 24 hr. [^{3}H] thymidine (2μ Ci/well) was added during the last 6 hr. Cell monolayers were washed with phosphate buffered saline and fixed with 10% trichloroacetic acid (TCA) solution. The TCA -precipitable material was dissolved in 0.5 ml of 0.3N NaOH containing 1% sodium dodecyl sulphate and radioactivity quantitated.
PKC enzyme activity: Quiescent PVSMC in 150 mm dishes in NG or HG were incubated for 15 min at 37^0 in media containing 0.2% BSA. Cells were washed and cell pellets sonicated in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 20 μg/ml leupeptin, 2 μg/ml aprotinin and 1 mM dithiothreitol. The lysates were centrifuged at 100,000xg for 1 hr. Supernatants (cytosol) were withdrawn and pellets resuspended in lysis buffer containing 0.1% NP-40, resonicated and saved as membrane fraction. PKC enzyme activity was assayed by phosphorylation of a PKC specific peptide substrate (10) using a PKC assay system from Gibco BRL.
12-HETE Measurement: Quiescent PVSMC in NG or HG in 100 mm dishes were incubated for 20 min at 37°C in fresh media containing 0.2% .BSA. 12-HETE levels in washed cell pellets were measured by our reported HPLC-radioimmunoassay method (11) after hydrolysis with methanolic potassium hydroxide.
Results are expressed as mean $\pm$ SE. Analysis of variance and students t test were used to compare control with experimental data. Duncan's test was also used for multiple comparisons.

RESULTS

Growth curves in NG and HG. Fig 1 represents the growth pattern of cells which have been through at least two passages in NG or HG DME media with 10% FCS. It is seen that the cells in HG grew nearly 40% faster than those in NG (1.42 $\pm$ 0.05 fold greater cell number in HG, $p<0.001$, $n=13$). In order to confirm that the changes observed were not secondary to changes in osmolality caused by the HG, the growth pattern of cells which had been through two passages in 5.5 mM glucose +19.5 mM mannose was studied. We found that cells cultured in mannose grew at a rate similar to those in NG and were also significantly slower than those in HG (1.3 $\pm$ 0.08 fold greater cell number in HG compared to mannose, $p<0.01$, $n=8$, results not shown).

The growth patterns of PVSMC cultured in NG or HG were also studied in very low serum conditions ie. media containing 0.2% BSA + 0.3% FCS and the results are shown in Fig. 2. Under these low serum conditions also a significantly increased growth rate was observed in cells cultured in HG (1.39$\pm$ 0.06 fold greater cell number in HG than NG, $p<0.01$, $n=9$).
Effect of elevated glucose on PVSMC protein and DNA synthesis. In order to further investigate the factors responsible for the increased growth rates in HG, the effects of HG on

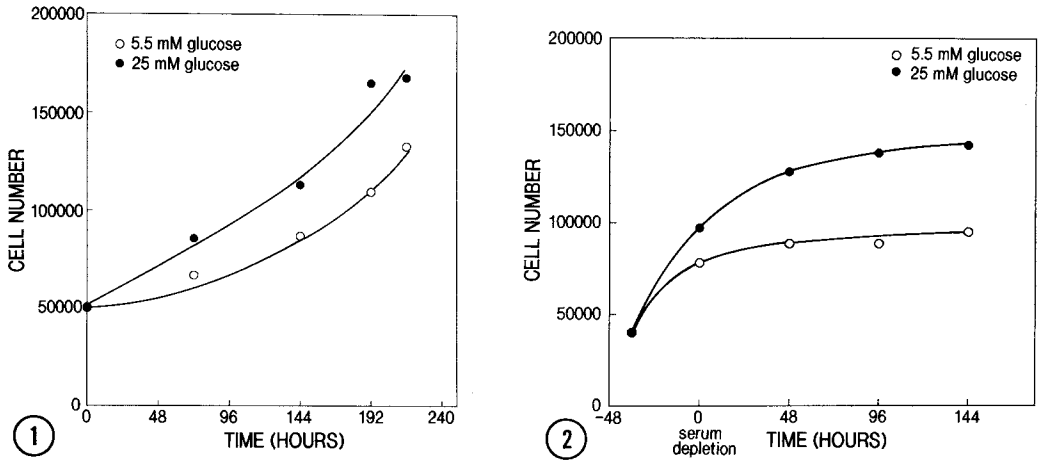

Fig. 1. Growth curves of PVSMC cultured for three passages in NG or HG. Cells were plated in medium containing 10% FCS and every 48 hr they were trypsinized and counted. Each point represents the mean of duplicate determinations. Similar results were obtained from three separate experiments. Cell numbers in HG were significantly greater than in NG ($p<0.001$) at all time periods tested.

Fig. 2. Growth curves of PVSMC cultured in NG or HG and low serum conditions. Cells were plated in media + 10% FCS for 24 hr. Medium was replaced with that containing 0.2% BSA + 0.3% FCS and cell counts obtained every 48 hr. Each point represents the mean of duplicate determinations. Similar results were obtained from three separate experiments. Cell numbers were significantly greater in HG than in NG ($p<0.01$) at all time periods tested.

PVSMC total cell protein and DNA synthesis were examined. Fig. 3 is a comparison of the FCS-induced DNA as well as protein synthesis in NG and HG. A significantly greater rate of both protein synthesis as well as DNA synthesis was observed in PVSMC cultured in HG compared to NG suggesting that elevated glucose has both hypertrophic and hyperplastic effects on PVSMC.

Effect of HG on PVSMC cell volume. In order to determine whether glucose-induced protein synthesis results in an increase in cell size, we examined the effect of HG on cell volumes using electronic cell volume measurements. Coulter counter measurements yielded cell diameters of 15.01 and 16.19 μm for NG and HG respectively which corresponds to a 1.25 fold greater mean cell volume in HG compared to NG. This volume difference was confirmed using a cell sorter. The results shown in Fig. 4 reveal that HG treatment caused a shift in the entire cell population to larger volumes with a change in the mean cell volume from 148 to 172 arbitrary volume units. Similar results were obtained in at least three separate experiments.

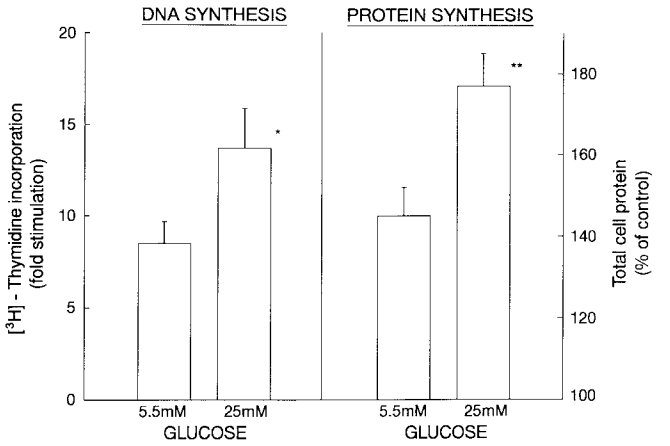

Fig. 3. Fetal calf serum - induced DNA synthesis and protein synthesis in PVSMC cultured in NG or HG. Confluent PVSMC in 12-well dishes were serum depleted for 24 hr and then treated with 10% FCS. DNA synthesis and total cell protein content were measured as described under Materials and Methods. Results are expressed as mean ± SE from seven separate experiments. * $p<0.02$; ** $p<0.05$ vs. NG.

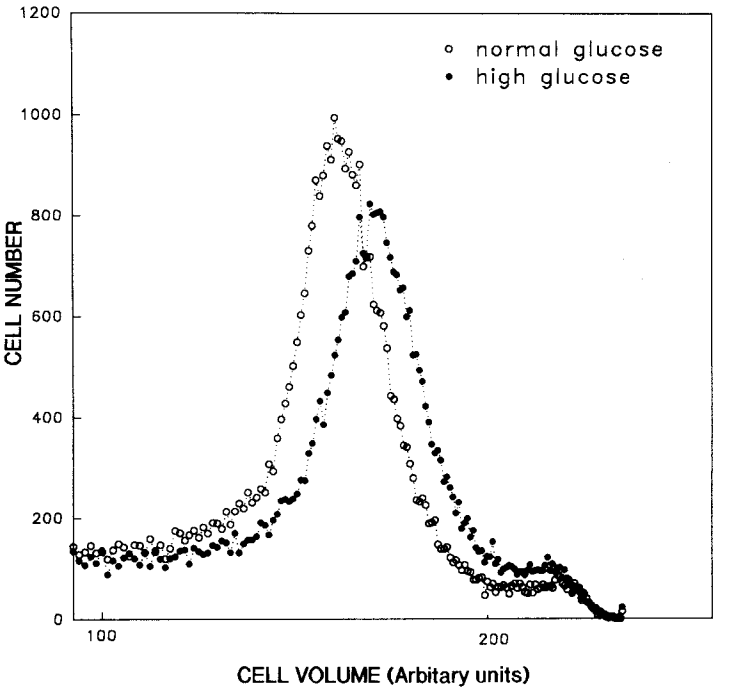

Fig. 4. Graph comparing PVSMC cell volume in NG and HG. Cells in T25 flasks were made quiescent for 24 hr and then incubated for 24 hr with NG or HG medium containing 10% FCS. Cells were harvested and analyzed on a cell sorter. The cell volumes shown in a log scale are typical of three such experiments.

TABLE 1

PKC activities in membrane and cytosolic fractions of PVSMC cultured in NG or HG

	PKC activity (pmol of ^{32}P incorporated/min/mg protein)	
	5.5 mM glucose	25 mM glucose
Cytosol	163 ± 12	128 ± 5*
Membrane	85 ± 10	150 ±15**

Results are expressed as mean ± SE from 5 separate experiments. Confluent PVSMC in NG or HG were serum depleted for 24 hr and then incubated for 15 min in fresh medium ± 0.2% BSA. PKC activity was measured as described under Materials and Methods.
* $P<0.03$; ** $P<0.01$.

PKC activity in NG and HG. In order to evaluate one potential mechanism for the increased growth of PVSMC in HG media, we examined PKC activity in quiescent PVSMC cultured in NG or HG. As shown in Table 1 membrane-bound PKC activities in HG were greater than in NG with a corresponding decrease in cytosolic PKC activities, thus suggesting that culturing cells in HG causes PKC activation.

12-HETE levels in NG and HG. We found that cells cultured in HG had significantly higher levels of cell associated 12-HETE compared to those in NG (2390±198 HG vs 1620±210 pg/10^6 cells NG, $p<0.01$). 12-HETE levels in cells cultured in mannose (1568±251 pg) were not significantly different from those cultured in NG.

DISCUSSION

In the present study, we have demonstrated for the first time that elevated glucose has both hypertrophic as well as hyperplastic effects on PVSMC in culture. Cells cultured in HG exhibited an increased growth rate both in 10% FCS as well as in low serum conditions. In order to more closely simulate chronic hyperglycemia, cells were passaged in HG rather than HG added acutely. The increased growth rates in HG were not due to changes in osmolality since cells cultured in high mannose grew significantly slower than those in HG. Further, FCS-induced protein synthesis (total cell protein) as well as DNA synthesis ([^{3}H] thymidine incorporation) were both elevated in HG. The increases in protein synthesis were accompanied by increases in cell volume. The effects of glucose on growth are not totally unexpected since recent evidence suggests that glucose stimulates growth in cells of *Saccharomyces cerevisiae* by inducing physiological events in the induction of new mitotic cell cycles and that these events

are cAMP-independent (12). These results suggest that hyperglycemia may be directly linked to accelerated vascular complications by increasing the growth rate of VSMC.

In evaluating potential mechanisms of accelerated growth rates in HG, we studied PKC activity in NG and HG. Several studies have demonstrated that HG increases PKC activity due to increased levels of diacylglycerol (5,13-17). Since PKC has several key cellular functions which include modulation of VSMC contraction and growth (6,18,19), it has been postulated that some of the vascular complications observed in diabetes may be a consequence of hyperglycemia - induced PKC activation (5). In the present studies, we have shown increased growth rates as well as increased PKC activity in PVSMC cultured in HG suggesting that PKC activation may be responsible, at least in part, for the accelerated growth produced by HG. However, more extensive studies will be required to confirm the involvement of PKC on glucose - induced mitogenesis since, most, but not all studies implicate PKC as a positive mediator of VSMC proliferation (6,19,20).

In the present report, we have also shown for the first time that HG conditions markedly stimulate the cellular production of the 12-LO product 12-HETE in PVSMC. Brown et. al. demonstrated that elevated glucose markedly augmented the production of 15-HETE by cultured vascular endothelial cells (21). Studies suggest that 12- and 15-HETE have mitogenic properties and can activate PKC and also certain oncogenes (7,8,22,23). Thus, increased lipoxygenase product formation in HG may mediate some of the increased growth effects of HG.

It is clear that there could be other mechanisms for the observed glucose-induced growth promoting effects such as induction of oncogenes and growth factors, imbalances in sorbitol metabolism or non-enzymatic glycosylation of cellular and extracellular products (24, 25). In addition, aminoguanidine has been shown to inhibit diabetic vascular dysfunction by blocking nitric oxide formation (26). Furthermore, increased synthesis of matrix components in high glucose (27,28) may be a contributing factor. Additional studies will be needed to evaluate these mechanisms and also whether there is a dose-dependent effect of glucose on growth.

Abnormal VSMC growth and proliferation are key events in the pathogenesis of hypertension and atherosclerotic vascular disease. Our novel observations suggest that

hyperglycemia may be linked to accelerated vascular disease by increasing smooth muscle cell growth and proliferation.

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