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Effect of high glucose on synthesis and gene expression of collagen and fibronectin in cultured vascular smooth muscle

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Abbreviations: FN, fibronectin; DMEM, Dulbecco's modified Eagle's medium; MEM, minimal essential medium; FBS, fetal bovine serum; NCP, noncollagenous protein

Abstract

Diabetes mellitus is an important risk factor of atherosclerosis. Although the mechanism for accelerated atherosclerotic disease in diabetes mellitus is not clear, extracellular matrix formation by vascular smooth muscle cells has been accepted to play important roles during development of atherosclerosis. High glucose condition has been reported to increase the synthesis of extracellular matrix such as collagen and fibronectin in cultured mesangial cells, implicating high glucose for the development of diabetic nephropathy. We studied here the effect of high glucose on the synthesis and gene expression of collagen and fibronectin in vascular smooth muscle cells. We found that vascular smooth muscle cells grown in high glucose (25 mM) medium for 1, 2, and 3 days synthesized more collagen and noncollagen protein, as compared to the cells grown in normal glucose (5.5 mM) medium for the same periods of time. We also found that cells grown in high glucose for 1, 2, and 3 days expressed higher level of type 1 procollagen mRNA than that of the cells grown in normal glucose. There was, however, no difference in the amount of newly synthesized fibronectin between cells grown in normal and high glucose condition. These results suggest hyperglycemic condition in diabetes may accelerate the atherosclerotic process by stimu-lating vascular smooth muscle cells to produce more collagen.

Keywords: vascular smooth muscle cell, collagen, fibronectin, high glucose, diabetes mellitus, atherosclerosis

Introduction

Diabetes mellitus is associated with a substantial increase in prevalence of atherosclerotic disease (Schwartz *et al*., 1992). However, the mechanisms for accelerated atherosclerotic disease in diabetes are unclear. It is widely accepted that the accumulation of extracellular matrix increases in the arterial intima during the development of atherosclerosis (Dodge and D'Amore, 1992; Ross, 1993; O'Brien and Chait, 1994). The extracellular matrix accumulated in atherosclerotic plaques consists of collagen, fibronectin (FN), elastin, glycosaminoglycans, and laminin, all of which are produced largely by vascular smooth muscle cells that have migrated from media, and partly by endothelial cells (Chamlet-Campbell and Campbell, 1981; Schwartz *et al*., 1992). As diffuse intimal thickening develops, type I and III collagen accumulate (O'Brien and Chait, 1994). In addition, it is suggested that type I collagen binds lipoproteins and its accumulation in the intima may promote lipoprotein accumulation (O'Brien and Chait, 1994).

Hyperglycemia *per se* in diabetes has been suggested to be a contributing factor to the development of the diabetic complications such as diabetic nephropathy and atherosclerosis (Ziyadeh *et al*., 1990; Spiro *et al*., 1995). For an instance, high glucose stimulates collagen and FN synthesis by cultured mesangial cells, suggesting an important role of hyperglycemia in the thickening of mesangium and basement membrane, a characteristic feature of diabetic nephropathy (Ayo *et al*., 1990; Studer *et al*, 1993; Ziyadeh *et al*., 1994).

Considering most of the collagen and FN accumulated in atherosclerotic plaques is produced by vascular smooth muscle cells, hyperglycemia might affect collagen and FN synthesis by vascular smooth muscle cells and contribute to the development of atherosclerosis in diabetes. Few studies, however, have addressed the direct effects of high glucose on collagen and FN synthesis in vascular smooth muscle cells. In the present study, we studied the synthesis and gene expression of collagen and FN by rat aortic vascular smooth muscle cells cultured under normal (5.5 mM) and high (25 mM) glucose conditions similar to normoglycemia and diabetic hyperglycemia, respectively.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Minimal essential medium (MEM), and fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, USA). Highly purified bacterial collagenase, trypsin, bovine serum albumin (BSA), ascorbic acid, dimethylsulfoxide, nonessential amino acid, salmon sperm DNA, formamide, agarose, antihuman fibronectin antibody, anti-goat IgG and Hoechst 33258 were from Sigma (St. Louis, U.S.A.). Sepharose 4B, gelatin-Sepharose 4B and protein A-Sepharose were from Pharmacia (Milwaukee, U.S.A.). Polystyrene microtiter plate and tissue culture plastic dishes were from Costar (Cambridge, U.S.A.). L-[5-^{3}H]proline (12.4 Ci/mmol), [^{35}S]methionine (1000 Ci/mmol) and [α-^{32}P]dCTP (3,000 Ci/mmol) were from Amersham (Arlington, U.S.A.). Random primed DNA labeling kit was from Boehringer Mannheim (Mannheim, Germany).

Cell cultures

A vascular smooth muscle cell line of rat, A-10 (CRL-1476), was obtained from American Type Culture Collection. Cells were cultured in DMEM supplemented with 20% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37˚C.

Measurements of collagen synthesis

Vascular smooth muscle cells were plated at 1×10^5 cells per well in 24-well culture plates and grown to confluence. Confluent cells were treated for 24 h with DMEM containing 2% FBS to go into the quiescent stage and the media was changed to fresh DMEM with a D-glucose concentration of 5.5 mM or 25 mM in 2% FBS. After conditioning for the indicated time, the synthesis of collagen and noncollagenous protein (NCP) in the cultured vascular smooth muscle cells were measured by the collagenase digestion method as described previously (Lee *et al*., 1995). Percent collagen synthesis, which represents the relative rate of collagen synthesis as a percentage of total protein synthesis, was then calculated by the formula of Peterkofsky *et al.* (1982):

Percent collagen synthesis =

$$\frac{\text{d. p. m. of collagen x 100}}{\text{(d. p. m. of NCP) x 5.4 + d. p. m. of collagen}}$$

where d. p. m. of NCP was determined by subtracting the d. p. m. of collagen from the d. p. m. of total protein.

Radioactive labelling and immunoprecipitation of FN

Confluent cells in 35-mm culture dishes were incubated with 2% serum medium in either normal (5.5 mM) or high glucose (25 mM) containing agents and then labeled for 2 h with methionine-free MEM containing 30 μCi/ml [^{35}S]methionine. Radiolabeled FN was immunoprecipitated as described previously (Lee *et al*., 1996). An excess (3 μg) of goat anti-human FN antibody was added to the combined samples of medium and cell layer extracts containing the same amount of radioactive protein and then incubated at 4˚C for 1-2 h. The immune complexes were recovered by the addition of 30 μl of protein A-sepharose beads (Pharmacia), and incubation with shaking for additional 1 h. Sepharose beads and adsorbed proteins were solubilized in 30 μl of electrophoresis sample buffer with heating at 100˚C for 4 min. Samples were analyzed by SDS-PAGE using a 6% gel, and subjected to fluorography.

Northern blot hybridization

Total cellular RNA was isolated with acid guanidinium thiocyanate-phenol-chloroform as described previously (Chomczynski and Sacchi, 1987). Ten μg of total cellular RNA per sample was fractionated by electrophoresis on a 0.8% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and followed by hybridization and subsequent washing as described by Sambrook *et al*. (1989). The cDNA probe was labeled with [α-^{32}P]dCTP using a random primed DNA labeling kit. The membrane was exposed to a X-ray film at -70˚C. The cDNA probe used for hybridization was HF677, human α1(I)procollagen cDNA (Chu *et al*., 1982). Autoradiograms were quantitated using the NIH image analysis program (Masters *et al*., 1992).

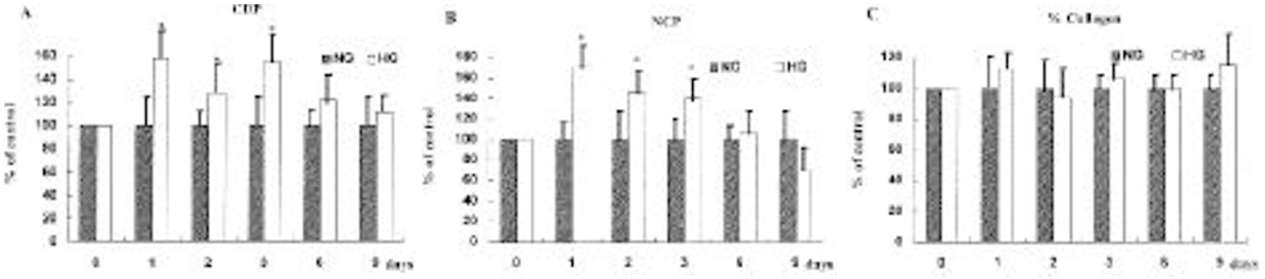
Statistical Analysis

Experimental data were analyzed by the Mann-Whitney test for comparisons of two groups. A value of $P < 0.05$ was considered to be significant.

Results

Effect of high glucose on collagen synthesis in vascular smooth muscle cells

To determine the effect of high glucose condition on collagen synthesis in vascular smooth muscle cells, cells were grown in the culture medium containing either normal (5.5 mM) or high (25 mM) glucose. Cells grown in high glucose medium for 1, 2, and 3 days synthesized 58%, 30% and 54% more collagen compared to the cells grown in normal glucose medium for the same periods of time, respectively, while cells grown in either normal or high glucose condition for 6 and 9 days synthesized similar level of collagen (Figure 1A). Cells grown in high glucose for 1, 2 and 3 days also synthesized 25%, 47%, and 40% more noncollagen protein compared to the cells grown in normal glucose for the



same periods of time, respectively, while thereafter there was no significant difference in the level of synthesis between normal and high glucose condition (Figure 1B). To determine a selective change in collagen synthesis, percent collagen synthesis which represents the relative rate of collagen synthesis as a percentage of total protein synthesis was calculated. Percent collagen synthesis did not differ significantly in the cells grown in either normal or high glucose condition (Figure 1C).

Figure 1. Effect of high glucose on the synthesis of collagen **(A)** and NCP **(B)**, and percent collagen synthesis **(C)** in vascular smooth muscle cells. Confluent quiescent cells were incubated with 2% serum medium containing [^{3}H]proline (2 μCi/ml) in either normal (5.5 mM) or high (25 mM) glucose condition for the indicated periods of time. Data are represented as mean ± SD of six measurements. * Significantly different from cells grown in normal glucose ($p < 0.05$). CDP, collagenase digestible protein; NG, normal glucose; HG, high glucose.

Effect of high glucose on the steady-state levels of a1(I) procollagen mRNA

To see if the observed increase in collagen synthesis is due to an induction of collagen mRNA, total RNA was isolated from the cells and analyzed by Northern blot. The steady-state levels of α1(I) procollagen mRNA, which is mRNA for the type I collagen, from the cells grown in high glucose for 1, 2 and 3 days were higher than those from the cells grown in normal glucose for the same periods of time (Figure 2A). To quantitate the mRNA levels further, the density of the collagen mRNA band shown in figure 2A was counted and normalized by that of 28 S rRNA, which was used as an internal control. Densitometric analysis of the mRNA bands showed 154%, 70% and 38% increase of α1(I) procollagen mRNA levels in the cells grown in high glucose for 1, 2 and 3 days compared with cells grown in normal glucose for the same periods of time, respectively (Figure 2B). There was no significant difference in the mRNA levels between cells grown in either normal or high glucose for 6 and 9 days (Figure 2B).

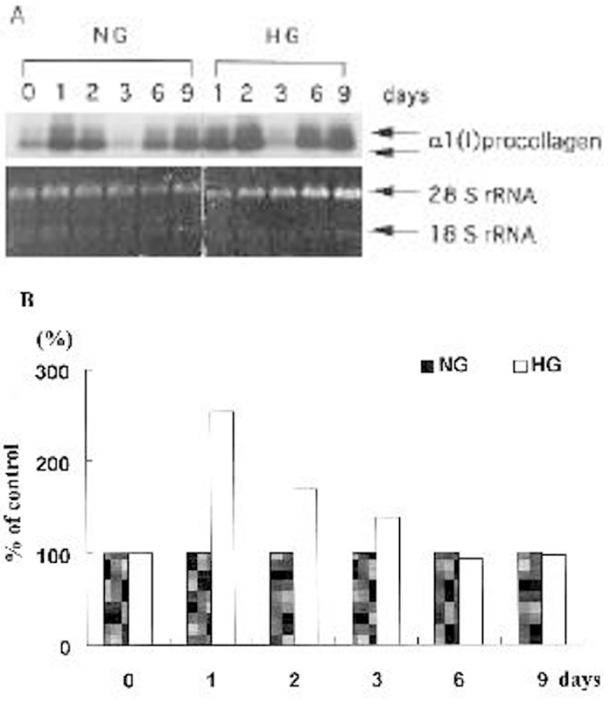


Figure 2. Effect of high glucose on the steady-state levels of α1(I) procollagen mRNA in vascular smooth muscle cells. **A**, Northern blot analysis of 10 μg of total RNA from the cells grown in either normal (5.5 mM) or high (25 mM) glucose for the indicated periods of time. The 4.3 kb α1(I) procollagen mRNA band is indicated. **B**, Densitometric analysis of the blot shown in the panel A. The density was quantitated using the NIH image analysis program. Each bar represents the density of the α1(I) procollagen mRNA bands after standardizing the amount of RNA in each lane with the 28 S rRNA signal. The values from high glucose are represented as a percent of those from normal glucose. NG, normal glucose; HG, high glucose.

Effect of high glucose on FN synthesis

Vascular smooth muscle cells grown in high glucose condition for 1-9 days did not show any significant difference in FN synthesis compared to the cells grown in normal glucose condition for the same periods of time, as shown in autoradiogram of immunoprecipitated fibronectin (Figure 3A) and in densitometric analysis of the fibronectin band (Figure 3B).

Discussion

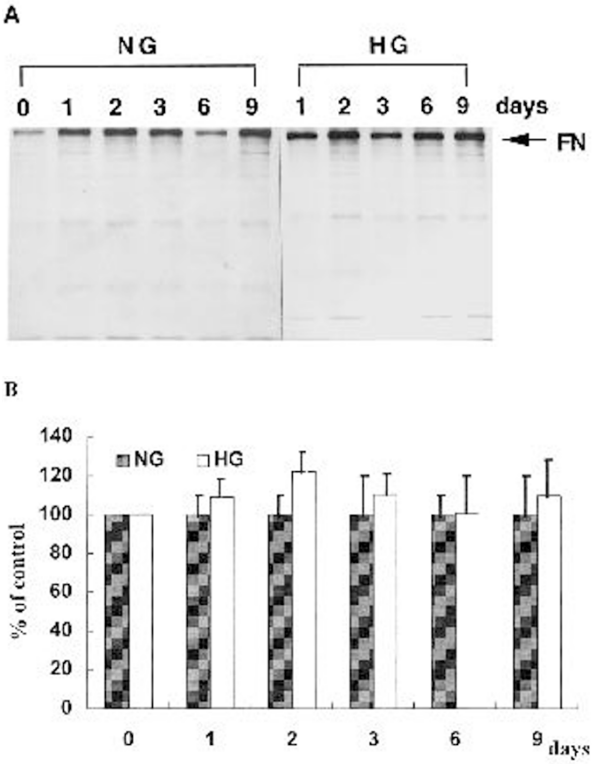


Figure 3. Effect of high glucose on FN synthesis in vascular smooth muscle cells. Confluent cells were incubated with 2% serum medium in either normal (5.5 mM) or high (25 mM) glucose for the indicated periods of time and then labeled with 30 μCi/ml [^{35}S]methionine for 2 h. **A**, FN was immunoprecipitated from the samples containing equal amount of radioactive protein and analyzed by SDS-PAGE. The FN band (molecular weight of 220 kDa) is indicated as an arrow. **B**, FN was immunoprecipitated from the samples containing equal amount of radioactive protein and the radioactivity (c.p.m.) of the solution containing FN was counted. Data are represented as mean ± SD of two experiments. NG, normal glucose; HG, high glucose.

Recent years have seen considerable progress in the elucidation of basic cellular and molecular mechanisms in the pathogenesis of atherosclerosis, the principal cause of heart attack, stroke and gangrene of extremities. The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to endothelium and smooth muscle of the artery wall. Plaque collagens, elastin, FN and proteoglycans are important in the pathogenesis of atherosclerosis (Schwartz *et al*., 1992; Raines *et al*., 1993; Ross, 1993). A large number of growth factors, cytokines and vasoregulatory molecules may participate in those process (Ross, 1993; O'Brien and Chait, 1994). That diabetes mellitus is a powerful risk factor for atherosclerosis is no longer in doubt, although the mechanisms through which this enhanced risk is expressed need clarification.

This study shows that vascular smooth muscle cells grown in high glucose condition synthesize more collagen and express more type 1 procollagen mRNA compared to the cells grown in the normal glucose condition. On the other hand, FN synthesis by cells grown in high glucose was not different from that by cells grown in normal glucose. These results suggest that hyperglycemia in diabetes may accelerate the development of atherosclerosis by stimulating vascular smooth muscle cells to produce more collagen and also can be a possible mechanism of enhanced risk of atherosclerosis in diabetes.

More than 10 types of collagen have been reported and known to have a tissue-specific distribution in vertebrate organisms (Miller, 1988). For example, type I collagen is the most prevalent type and distributes ubiquitously in many tissues including dermis, bone, and blood vessel, while type IV collagen is a major constituent of basement membranes (Miller, 1988). Type analysis of collagen synthesized by cultured arterial smooth muscle cells has reported that arterial smooth muscle cells synthesize types I, III, IV, and V collagen and of these, type I collagen is the predominant type of collagen (Layman *et al*., 1977; Okada *et al*., 1993). Furthermore, collagen type I is the major type in the atherosclerotic plaque as in the normal vessel wall (Barnes, 1988). Thus, we focused on the alteration in the expression of type I collagen among the collagen types reported to be synthesized by vascular smooth muscle cells. We observed the increase of total collagen regardless of collagen types in response to high glucose by collagenase digestion method since this method does not distinguish the type of collagen. Then, we observed the increase of type I collagen mRNA levels by high glucose in Northern blot analysis.

Previous investigators have demonstrated that high glucose concentration stimulates type I and IV collagen synthesis and increases type I and IV procollagen mRNA levels in murine mesangial cells and cortical tubule cells (Ziyadeh *et al*., 1990). High glucose has also been shown to increase collagen synthesis and type IV and VI procollagen mRNA levels in rat heart endothelial cells (Spiro *et al*., 1995) and increase collagen synthesis in bovine retinal capillary pericytes (Li *et al*., 1984). In addition to collagen, high glucose stimulated FN synthesis in rat and human glomerular mesangial cells (Rasmussen *et al*., 1989; Nahman *et al*., 1992; Kreisberg *et al*., 1994) and also in rat and human endothelial cells (Roy *et al*., 1990; Spiro *et al*., 1995). In our study, high glucose stimulated collagen synthesis and type I procollagen mRNA expression in rat vascular smooth muscle cells, while it did not stimulate FN synthesis in the cells. The different response of FN synthesis between mesangial cells and vascular smooth muscle cells may reflect the tissue-specificity of FN expression in response to high glucose. In this regard, we and others have reported the

tissue-specific expression of FN in response to the activation of protein kinase C or the elevation of intracellular cAMP level (Dean *et al*., 1989; Lee *et al*., 1996). The mechanism of the tissue-specific expression of FN still remains unknown. In addition, the stimulation of collagen synthesis observed in the cells grown in a high glucose condition for 1, 2, and 3 days returned to normal level after culture for 6 and 9 days and the mechanism of the lack or difference of response to high glucose after culture for 6 days is still unclear. However, vascular smooth muscle cells cultured for more than 6 days in high glucose condition become too confluent compared to the cells cultured for same period in normal glucose condition. We think it could be a contributing factor for the lack or difference of response.

Recently, there have been many studies about the local factors and mechanisms involved in the stimulation of collagen and FN by high glucose. Some of the studies have demonstrated that autocrine activation of transforming growth factor-β (TGF-β) mediates the stimulation of collagen gene expression in murine mesangial cells by high glucose (Ziyadeh *et al*., 1994; Sharma *et al*., 1996). In addition, TGF-β has been shown to stimulate collagen synthesis in human vascular smooth cells suggesting its role in high glucose stimulation of collagen synthesis (Okada *et al*., 1993). A Recent study has shown that another growth factor, platelet derived growth factor-β, can be involved in the development of diabetic angiopathy by high glucose (Inaba *et al*., 1996). Protein kinase C (PKC), a major component of intracellular signal pathway, has been reported to play a role in the increased FN accumulation by mesangial cells grown in high glucose (Craven and DeRubertis, 1989; Studer *et al*., 1993). Furthermore, high glucose has been shown to stimulate FN gene expression in mesangial cells through a cAMP response element (Kreisberg *et al*., 1994; Kreisberg and Kreisberg, 1995), suggesting the involvement of the cAMP-dependent signal pathway. Whether these factors and signal pathways participate in the stimulation of collagen synthesis in vascular smooth muscle cells by high glucose deserves additional studies.

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