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Examination of Monocyte Adherence to Endothelium Under Hyperglycemic Conditions

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Increased nonenzymatic glycation of proteins has been implicated in the pathogenesis of diabetic vascular disease. The authors have shown by ^{3}H-$NaBH_4$ reduction of nonenzymatic glycation adducts that endothelial cell membrane proteins undergo increased nonenzymatic glycation *in vitro* ***when exposed to elevated concentrations of glucose. Increased nonenzymatic glycation also was found*** *in vivo* ***for microvascular endothelial cells isolated from streptozotocin-induced diabetic rats compared with control rats. Cultured monocytes have previously been reported to express receptors for certain nonenzymatic glycation adducts. The authors have further investigated whether monocyte interactions with endothelium are altered by the presence of nonenzymatic glycation adducts on endothelium. Adherence assays were performed in the presence of elevated concentrations of glucose with decreased NaCl levels to maintain normal osmolarity (as occurs physiologically). Although monocyte adherence to endothelium and levels of early nonenzymatic glycation adducts increased under these conditions, the increased adherence appears to be due to the altered NaCl levels. In fact, freshly isolated monocytes (in contrast to what has been found for macrophages and activated monocytes) were shown not to express appreciable numbers of receptors for nonenzymatic glycation adducts. (Am J Pathol 1991, 139:1089–1097)***

The role of hyperglycemia in the development of vascular complications in diabetes is unclear. There is growing evidence, however, that high blood levels of glucose in diabetics can cause pathologic changes in many plasma proteins through the process of nonenzymatic glycation.[1] The reaction between glucose and the amino groups of proteins proceeds through a series of steps.[2] When in chain form, the carbonyl group of glucose can undergo a nucleophilic attack by an amino group to produce an unstable, reversible, nonenzymatic glycation adduct (an aldimine). This reaction is very rapid, and the concentration of the adduct is influenced by *actual* blood glucose levels. The second step is slow and leads to the formation of a relatively stable ketoamine. It accumulates as a function of all the blood glucose levels over the lifetime of a given protein and therefore reflects *past* glucose concentrations. After very long periods (physiologically, after months or years), the ketoamine slowly rearranges itself to form advanced irreversible structures.

Methods used to quantitate certain nonenzymatic glycation adducts (ketoamines) have detected twofold to threefold increases in the levels formed on diabetic hemoglobin, the best known example of such modified proteins.[3] Similar increases have been shown to occur on a wide variety of plasma proteins and interstitial matrix proteins.[4–14] The presence of increased nonenzymatic glycation adducts at critical points in a protein sequence can alter the normal function of the protein. It has been shown *in vitro* that such properties as enzyme activity,[15] the binding of regulatory molecules,[16] the cross-linking of proteins,[7] protein susceptibility to proteolysis,[1] macromolecular recognition and endocytosis,[17] and immunogenicity[18] can be altered by increased nonenzymatic glycation.

Although modification of the endothelial cell surface by nonenzymatic glycation adducts has not been adequately studied, increased nonenzymatic glycation could alter some of the normal functions of the endothelium and thereby contribute to the development of vascular disease in diabetes. The interactions between endothelial cells and monocytes, in particular, could be affected. It has recently been found that cultured monocytes possess receptors for advanced nonenzymatic glycation end products formed over time from the early, reversible adducts,[18] and they are able to recognize, bind, and

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ingest a variety of proteins with such adducts.[19] As monocyte adhesion to endothelium has been shown to be an early step in the development of atherosclerotic lesions,[20] it is important to investigate whether elevated concentrations of glucose result in increased nonenzymatic glycation of the endothelial cell surface and whether such modification affects the interactions of blood monocytes with endothelium.

Materials and Methods

Endothelial Cell Isolation and Culture

Bovine pulmonary arteries were flushed with Hank's balanced salt solution (HBSS) with 10 mmol/l (millimolar) HEPES, pH 7.4 (buffered HBSS) with antibiotics, incubated in 0.1% collagenase at 37°C for 20 minutes, and scraped with a rubber policeman.[21] The lung tissue was minced, suspended in 0.1% collagenase, and incubated at 37°C for 1 hour.[22] After centrifugation at 300*g* for 5 minutes, the cells were suspended in Dulbecco's modified Eagle's medium with 5% supplemented calf serum (SCS, Gibco Laboratories, Grand Island, NY), 5% NuSerum (NS, Collaborative Research, Waltham, MA), and antibiotics and subsequently plated. Endothelial cell colonies were initially identified and selected on the basis of their cobblestone morphology,[23] positive immunofluorescence staining for factor VIII,[24] and the binding and uptake of fluorescently labeled acetylated low-density lipoprotein (LDL).[25]

In vivo studies entailed the isolation of microvascular endothelial cells from the epididymal fat pads of streptozotocin-induced diabetic rats. The distal two thirds of the epididymal fat pads were removed 2 weeks after injection, dissected, and minced to the consistency of a paste. Samples from 5 normal and 10 diabetic rats were pooled for each determination. (More diabetic rats were required because they had less fat than normal rats.) After centrifugation (100*g*, 7 minutes, 4°C), the vascular pellet was resuspended in buffered HBSS with 0.2% collagenase and incubated for 40 minutes at 37°C. The pellet then was washed in phosphate-buffered saline, resuspended in 45% (vol/vol) Percoll, and centrifuged (23,000*g*, 15 minutes, 4°C). Verification of the presence of microvessels was made with phase-contrast microscopy.[17]

Isolation of Endothelial Cell Membranes

Endothelial cells were suspended in 10 mmol/l TRIS-HCl buffer (pH 7.6) with 0.005% phenylmethylsulfonyl fluoride, 1 mmol/l diisopropyl fluorophosphate, 0.2 mmol/l 1,4-dithio-L-threitol, and 1 μg/ml leupeptin (protease inhibitors). After sonication, the suspension was centrifuged twice at 2000*g* for 6 minutes. Then the pellet was resuspended and centrifuged at 12,000*g* for 10 minutes. The resulting supernatant was combined with the top white layer of the pellet and centrifuged at 100,000*g* for 69 minutes to produce the total-membrane pellet.[26,27]

Measurement of Nonenzymatic Glycation

Endothelial cell membranes were exposed to various concentrations of glucose for 3 weeks at 37°C. After washing, 1 mmol/l ^{3}H-NaBH$_4$ was added to reduce nonenzymatic glycation adducts.[28–30] The membrane proteins then were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis,[31] the gel was stained with Coomassie blue, and each lane was cut into 24 slices. The gel slices were dissolved overnight in 30% H_2O_2 at 50°C, and ^{3}H incorporation was measured by scintillation counting. Additionally endothelial cells from rat epididymal fat pads were isolated as described above; however, 1 mmol/l ^{3}H-NaBH$_4$ was added after the first centrifugation, and the vascular pellet was subsequently washed thoroughly. After isolation of the microvessels, aliquots were taken for scintillation counting and protein determination using the BCA Protein Assay Reagent (Pierce).

Monocyte Adherence to Endothelium

Monocytes were separated from whole human blood using Sepracell-MN (Sepratech Corporation, Oklahoma City, OK).[32] Purity was 84% as assessed by nonspecific esterase staining. When endothelial cells were preincubated with high concentrations of glucose, the culture medium (Basal Media Eagle, Gibco, with 5% SCS, 5% NS, and antibiotics) was osmotically balanced by reducing NaCl levels an equivalent number of milliosmoles. When high concentrations of glucose, mannitol, or sorbitol were present during adhesion assays, monocytes were likewise suspended in osmotically balanced media. Isolated monocytes were labeled at 4°C for 1 hour with chromium-51 (100 μCi/ml cell suspension, New England Nuclear, Boston, MA), rinsed, and resuspended in the above medium at a concentration of 2×10^5 cells/ml. The cells were subsequently added to confluent monolayers of endothelial cells and incubated at 37°C for 45 minutes.[33] The percentage of adherent cells was quantitated by scintillation counting.

Monocyte Adherence to Protein and Plastic Substrate

Bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) was nonenzymatically glycated by suspend-

ing it at a concentration of 1% in phosphate-buffered saline (PBS) and incubating at 37°C for 3 weeks in the presence of 200 mmol/l D-glucose. Control BSA was treated similarly. Round glass coverslips (15 mm diameter) were soaked overnight in the above suspensions and rinsed thoroughly. Additionally polystyrene dishes were treated with 70% perchloric acid and saturated potassium chlorate solution in a 3:2 (vol:vol) ratio for 10 minutes, followed by esterification with mildly acidic N-butanol to increase selectively the hydroxyl residue concentration on the polystyrene surface.[34] Two hundred microliters of a suspension of ^{51}Cr-labeled monocytes (1×10^6 cells/ml) was added to coverslips or treated polystyrene dishes. After incubation at 37°C for 30 minutes, the percentage adherence was quantitated by scintillation counting.

Binding Experiments

Glycated BSA (GlyBSA) and control BSA were radioiodinated with carrier-free ^{125}I (NEN) by the IODO-GEN method of Fraker and Speck[35] and recovered by trichloroacetic acid precipitation. Specific activities for BSA and GlyBSA were 3.04×10^4 cpm/ng and 3.26×10^4 cpm/ng, respectively. Monocytes were isolated as described above, seeded onto plastic tissue-culture dishes, and allowed to adhere for 2 hours. After extensive washing, binding was initiated by removing the wash medium and adding 200 μl incubation medium containing the radioligand. Binding was carried out at 4°C for 2 hours, with gentle agitation. (To evaluate binding in 5 mmol/l or 100 mmol/l glucose, the osmotically balanced media described above were used. Gel filtration of BSA in 5 mmol/l and 100 mmol/l glucose was performed to show that no aggregation of BSA occurred in 100 mmol/l glucose.) Cell-associated radioactivity was measured by scintillation counting, and any specific binding observed with control BSA was subtracted from total binding in determining the specific binding of GlyBSA.

Statistics

The Wilcoxon rank sum test was used for measurements of nonenzymatic glycation. The Student's unpaired *t*-test was used for each of the monocyte adherence assays.

Results

To determine whether endothelial cell membrane proteins undergo increased nonenzymatic glycation when exposed to elevated concentrations of glucose, endothelial cell membranes were isolated and incubated in buffer containing 5 mmol/l, 20 mmol/l, or 100 mmol/l glucose for 3 weeks. Nonenzymatic glycation adducts then were measured by reduction with ^{3}H-NaBH$_4$. As shown in Figure 1, virtually all of the endothelial cell membrane protein fractions underwent increased nonenzymatic glycation when exposed to elevated concentrations of glucose. Although this represents nonenzymatic glycation of total membrane preparations, it suggests that the luminal surface, as well as other portions of membrane, undergo such modification.

To determine whether such increases could also be found *in vivo,* microvascular endothelial cells were isolated from the epididymal fat pads of normal and streptozotocin-induced diabetic rats at 2 weeks after injection. Figure 2 shows the relative levels of nonenzymatic glycation adducts found on the external surfaces of the cells as measured by ^{3}H-NaBH$_4$ reduction compared with the blood glucose levels of the rats at the time of death. Amounts of nonenzymatic glycation were found to increase roughly in proportion to blood glucose levels.

Cultured monocytes are known to have receptors for certain nonenzymatic glycation adducts. Because endothelium has now been shown to undergo increased nonenzymatic glycation when exposed to high concentrations of glucose, it was important to determine whether receptor-mediated recognition of nonenzymatic glycation adducts would increase the adherence of blood monocytes to endothelium. As monocytes isolated from different donors or from the same donor on different days showed variations in control values for monocyte adhesion, ranging from 10.2% to 60.8%, all adhesion assays were performed simultaneously with controls, and results are expressed as percentage of control values. As shown in Figure 3, preincubation of endothelium for up to 6 days in media containing 100 mmol/l glucose and decreased NaCl to maintain normal osmolarity resulted in no difference in subsequent monocyte adherence. When the assay was performed with no preincubation and in the presence of the high glucose/low NaCl media, however, a 75% increase in monocyte adherence was observed. These results suggested that the late irreversible nonenzymatic glycation adducts were not associated with increased monocyte adhesion, but that the early reversible adducts may have been.

The high glucose/low NaCl media appeared not to activate the monocytes and thereby increase adhesion in general, as no increase was seen in monocyte adherence to tissue-culture plastic alone (Figure 3). When monocyte adhesion to endothelial monolayers was measured in the presence of 20 mmol/l glucose (and decreased NaCl levels to maintain normal osmolarity), a smaller increase was found, although it was not statistically significant.

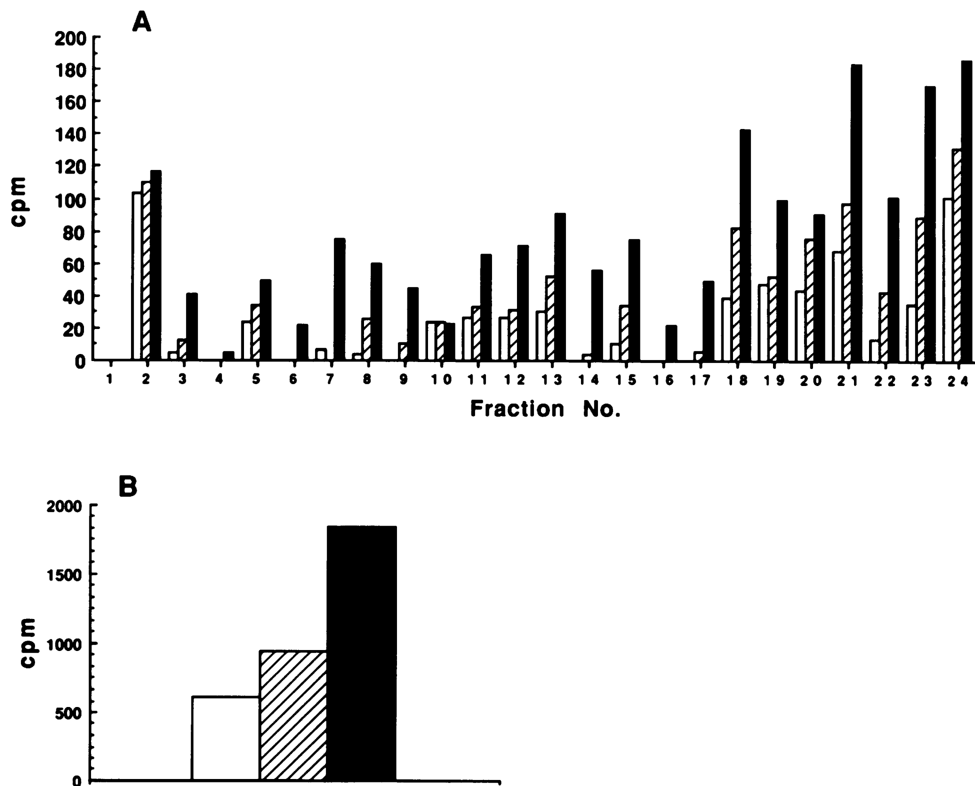

Figure 1. *Nonenzymatic glycation of bovine pulmonary artery endothelial cell membrane proteins following incubation in 5 mmol/l (□), 20 mmol/l (▨), or 100 mmol/l (■) glucose for three weeks. Nonenzymatic glycation adducts were reduced with ^3H-$NaBH_4$, and proteins were separated using SDS-PAGE. Each lane was sliced into 24 fractions, and ^3H incorporation was determined by scintillation counting of dissolved gel fractions. Values are expressed for each of the 24 fractions* (A) *and for the sum of all fractions for each sample* (B).

To determine whether early nonenzymatic glycation adducts can form on the endothelial cells in the amount of time required for the monocyte adherence assay, endothelial cell monolayers were incubated for only 30 minutes in media containing 5 mmol/l or 100 mmol/l glucose. Surface adducts were then reduced with ^3H-$NaBH_4$, and radioactivity was measured by scintillation counting. Cells exposed to 100 mmol/l glucose were found to incorporate 162 ± 12 cpm/μg protein compared with 124 ± 5 cpm/μg protein of controls, reflecting a 31% increase in surface nonenzymatic glycation ($n = 3$, $P = 0.05$).

If early nonenzymatic glycation adducts mediate the

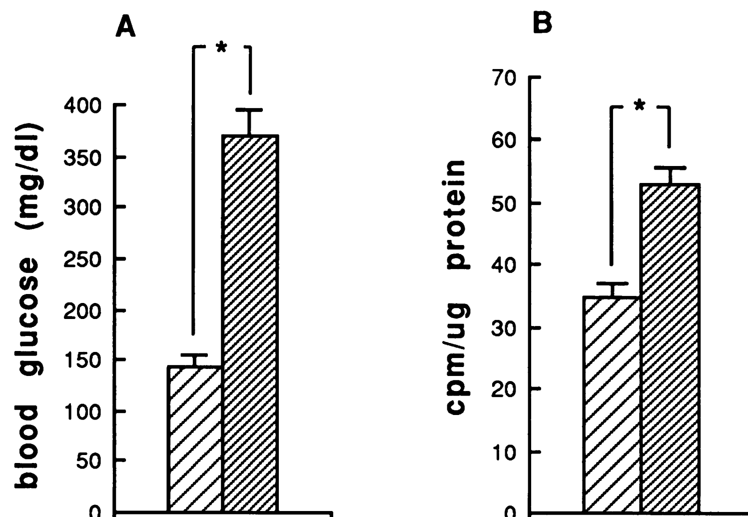

Figure 2. A: *Blood glucose levels of normal (▨, n = 30) and streptozotocin-induced diabetic (▨, n = 15) rats at the time of sacrifice.* B: *Nonenzymatic glycation of microvascular endothelial cells isolated from the epididymal fat pads of normal (▨) and diabetic (▨) rats. Each determination (n = 3) made with endothelial cells pooled from 5 normal or 10 diabetic rats. Error bars represent SEM.* *P = 0.05.

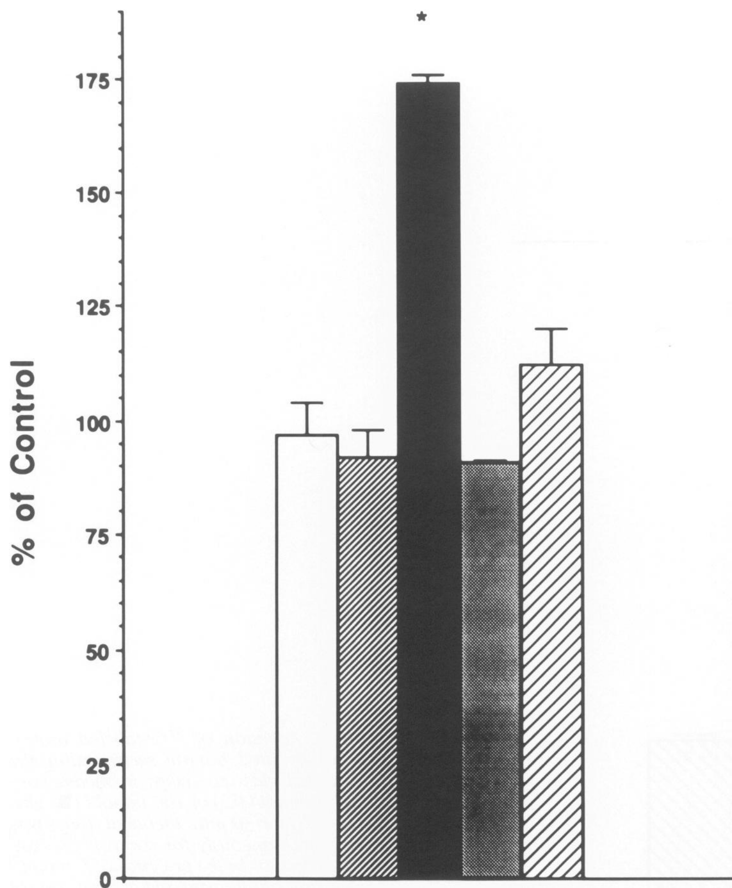

Figure 3. *Adhesion of ^{51}Cr-labeled monocytes in buffer to endothelial cell monolayers at 37°C for 40 minutes following 2 (□) or 6 (▨) days of preincubation in media containing 100 mmol/l glucose. Adhesion also performed over endothelial cells (■) and tissue-culture plastic (▩) in media containing 100 mmol/l glucose with no pre-incubation, and over endothelial cells in media containing 20 mmol/l glucose (▨) with no pre-incubation. Identical assays performed simultaneously for controls with 5 mmol/l glucose, and all values expressed as % of control values. Error bars represent SEM. n = 3, *P = 0.001.*

increase in monocyte adhesion to endothelium observed in the presence of elevated concentrations of glucose, one would predict that the same effect would occur if the assay were performed over a protein substrate rather than an endothelial monolayer. Bovine serum albumin was chosen as the protein substrate because monocytes normally have very low affinity for it, and it is known to undergo increased nonenzymatic glycation in the presence of high concentrations of glucose.[4] Figure 4 shows that only a very small increase occurred in monocyte adhesion to BSA that had been preincubated for 3 weeks in 200 mmol/l glucose (glycated BSA). When the assay was performed in the high glucose/low NaCl media, however, monocyte adhesion increased to almost three times the control value. Again the high glucose/low NaCl media appeared not to activate the monocytes such that adhesion in general was increased, because no increase was observed in monocyte adhesion to glass alone.

To determine whether simply increasing the hydroxyl residue density on a substrate affects subsequent monocyte adherence, polystyrene dishes were chemically treated to increase selectively the hydroxyl residue density, and monocyte adherence to treated and untreated dishes was measured. After 15 minutes at 37°C, 18.4 ± 1.6% of monocytes adhered to control polystyrene, whereas 24.1 ± 0.9% of cells adhered to the treated polystyrene ($n = 4$, $P < 0.05$).

To make sure that the decreased NaCl levels did not affect monocyte adherence to endothelium under the high glucose/low NaCl conditions, further adhesion assays were performed using high mannitol/low NaCl or high sorbitol/low NaCl. Unexpectedly, as shown in Figure 5, the same effects were observed in the experiments with mannitol and sorbitol as were found with glucose. Therefore, although nonenzymatic glycation adducts simultaneously increased on endothelium and increased hydroxyl group density was shown to increase monocyte adherence, the increased adherence of monocytes to endothelium we observed under high glucose/low NaCl conditions appeared to be a result of the low NaCl.

Additionally freshly isolated blood monocytes were shown not to possess significant numbers of receptors for nonenzymatic glycation adducts, as opposed to what has previously been found for cultured monocytes. As shown in Figure 6A, no specific binding of ^{125}I-GlyBSA to freshly isolated monocytes was observed. Nevertheless Figures 6B and 6C show effects that were observed when the binding studies were performed in the pres-

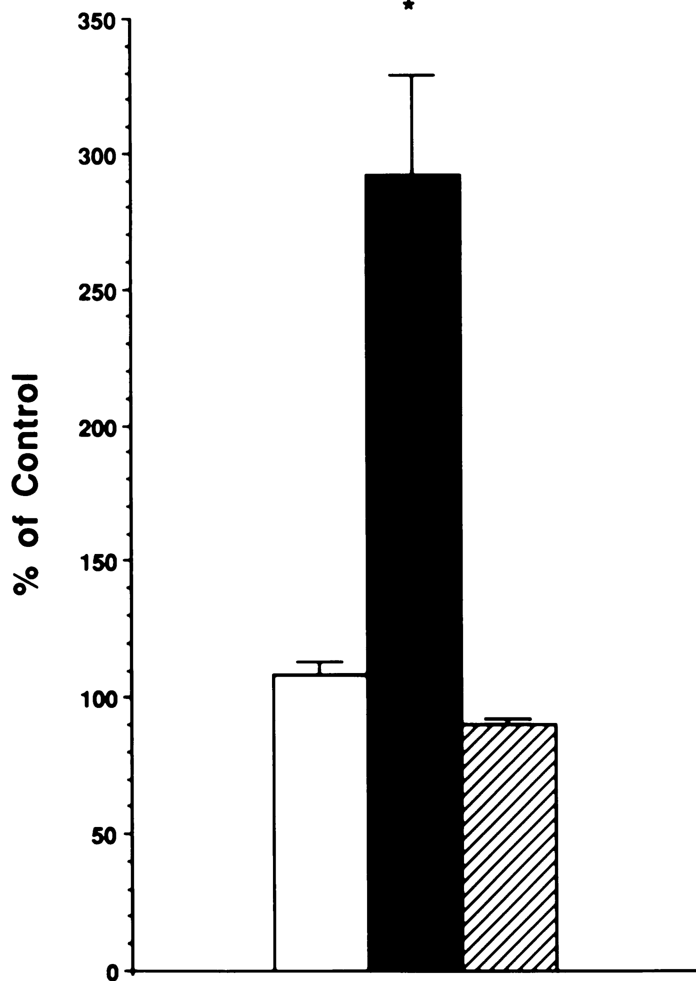

Figure 4. *Adhesion of ^{51}Cr-labeled monocytes to glycated bovine serum albumin (BSA)-coated glass coverslips in media containing 5 mmol/l (□) of 100 mmol/l (■) glucose at 37°C for 30 min. Identical assays performed simultaneously for controls on BSA-coated coverslips in the presence of 5 mmol/l glucose, and results expressed as % of control values. Adhesion to noncoated glass coverslips in the presence of 100 mmol/l glucose (▨) also compared to controls in 5 mmol/l glucose. Error bars represent SEM. n = 3, *P < 0.05.*

ence of normal or high glucose/low NaCl media. The data points represent nonspecific binding of BSA (B) and GlyBSA (C). In each case, although no specific binding was found, nonspecific binding increased in the presence of the high glucose/low NaCl media.

Discussion

High concentrations of glucose were found to increase the nonenzymatic glycation of endothelial cell membrane proteins *in vitro* and *in vivo,* and increased monocyte adherence to endothelium was observed under hyperglycemic conditions (high glucose/low NaCl). As monocyte adhesion to tissue-culture plastic and glass did not increase under similar conditions, it appeared that the high glucose/low NaCl media did not activate monocytes and thereby increase their adhesive properties in general. Nor does the up-regulation of monocyte receptors for endothelial cell adhesion molecules or the increased expression of endothelial cell adhesion molecules in the presence of high glucose/low NaCl adequately explain the increased monocyte adhesion we observed, as we found the same effect when monocytes were allowed to adhere to an albumin substrate in the presence of the high glucose/low NaCl media. It initially appeared, therefore, that increased monocyte adherence to the protein substrate in the high glucose/low NaCl media may have been due to a modification of the substrate by glucose. The modification known to occur on both the endothelial cell surface and the albumin substrate during the time required for the monocyte adhesion assay is the formation of early nonenzymatic glycation adducts.

Other data had suggested that a high density of hydroxyl groups on a given molecule is sufficient to allow it to act as a substrate for cell adhesion.[36] Tissue-culture grade polystyrene dishes are prepared by a method that produces carbonyl and hydroxyl groups on the surface.[37] Blocking carbonyl groups does not affect cell adhesion, but blocking hydroxyl groups severely inhibits cell adhesion. Increasing the density of hydroxyl groups

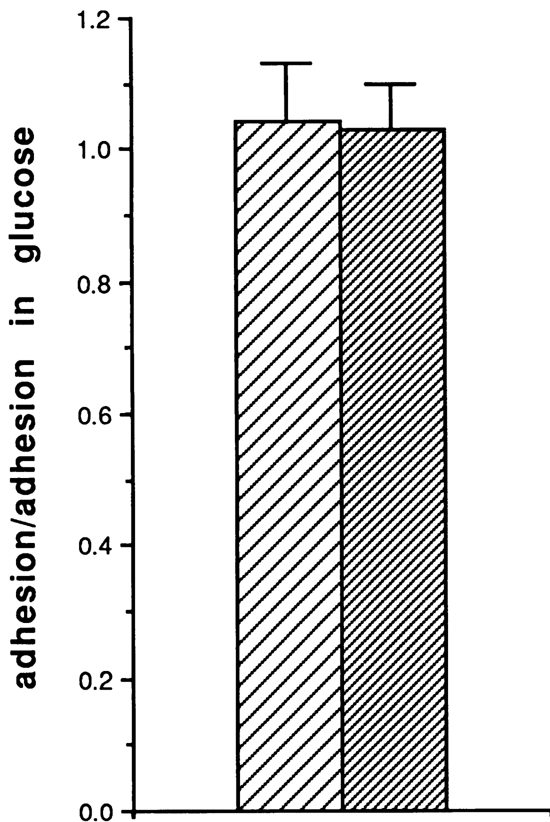

Figure 5. *Adhesion of* ^{51}*Cr-labeled monocytes to endothelial cell monolayers in the presence of 100 mmol/l mannitol or 100 mmol/l sorbitol at 37°C for 40 minutes. Results are compared with adhesion in the presence of 100 mmol/l glucose under the same conditions. Error bars represent SEM.* $n = 3$, P = NS.

on the surface causes cells to adhere to an even greater extent.[34] A number of substrates that possess a high degree of hydroxylation are known to enhance cell adhesion, including polyserine adhered to glass or polystyrene, partially-hydrolyzed polyvinylacetate, hydroxylated polypropylene, and hydroxylated polybut-1-ene.[34] An increase in the nonenzymatic glycation of endothelial cell surface proteins would similarly increase the density of hydroxyl groups present, and our data showed that increasing the density of hydroxyl groups on polystyrene resulted in increased monocyte adherence.

Alternatively it appeared that the increase in monocyte adhesion to endothelium might have resulted from a change in the charge of endothelial cell surface proteins due to nonenzymatic glycation. When the amino groups of hemoglobin beta-chains undergo nonenzymatic glycation, there is a significant charge change (due to a reduction in the negative log of dissociation constant [pKa] value) that enables the HbA_{Ic} moiety to be detected in analytic methods based on charge properties. The earliest nonenzymatic glycation adducts (aldimines) are not detected by such methods because they dissociate when not directly exposed to elevated concentrations of glucose.[31] Because aldimine formation requires that the amino group not be protonated, however, the aldimines would be expected to have altered charge properties as well.

Subsequent experiments to control for the decreased NaCl by using high mannitol/low NaCl and high sorbitol/low NaCl media, however, showed that the same increase in monocyte adherence to endothelium occurred under these conditions as was observed using the high glucose/low NaCl media. As mannitol and sorbitol do not form nonenzymatic glycation adducts, it appeared that the low NaCl was responsible for the increased monocyte adherence. In fact, it was found that the freshly isolated monocytes do not even express an appreciable number of receptors for nonenzymatic glycation adducts, as opposed to what has been previously reported for cultured monocytes and macrophages.[18]

Although our data do not rule out the possibility that nonenzymatic glycation adducts affect monocyte adherence to endothelium, it suggests that low NaCl is primarily responsible for the changes we have observed. Decreased plasma levels of NaCl are known to occur physiologically during hyperglycemia, as increased renal excretion of NaCl is important to maintain normal osmolarity.[38] Other researchers have found that changes in the ionic strength of the medium of similar magnitude to those occurring in these studies can affect cell adhesion to polystyrene, glass, collagen, and fibronectin.[39,40] The altered adhesion may be accounted for by the effects of electrolytic properties of the medium on electrostatic repulsive interactions between the interacting cells.[40] The findings may be explained in particular by the Derjagun–Landau–Verwey–Overbeck (DLVO) theory of cell adhesion.[41] The same principle may explain why we observed increased nonspecific binding of BSA to monocytes in the presence of the high glucose/low NaCl media.

Changes in NaCl levels of plasma occurring during hyperglycemic episodes in diabetes are not usually as large as were used in these studies. Smaller changes in NaCl levels may have similar but smaller effects on monocyte adherence to endothelium, but small changes in monocyte adherence are not detectable with our assay. Nevertheless small changes in the ionic strength of plasma may be very important physiologically if they occur over a long period. Although one would expect large changes in monocyte adhesion to result in relatively acute manifestations of disease, atherosclerosis and small vessel disease in diabetes are very slow processes requiring many years to develop. A relatively small but chronic perturbation of monocyte–endothelial cell interactions, therefore, appears particularly relevant to the pathogenesis of slowly progressive vascular disease in diabetes.

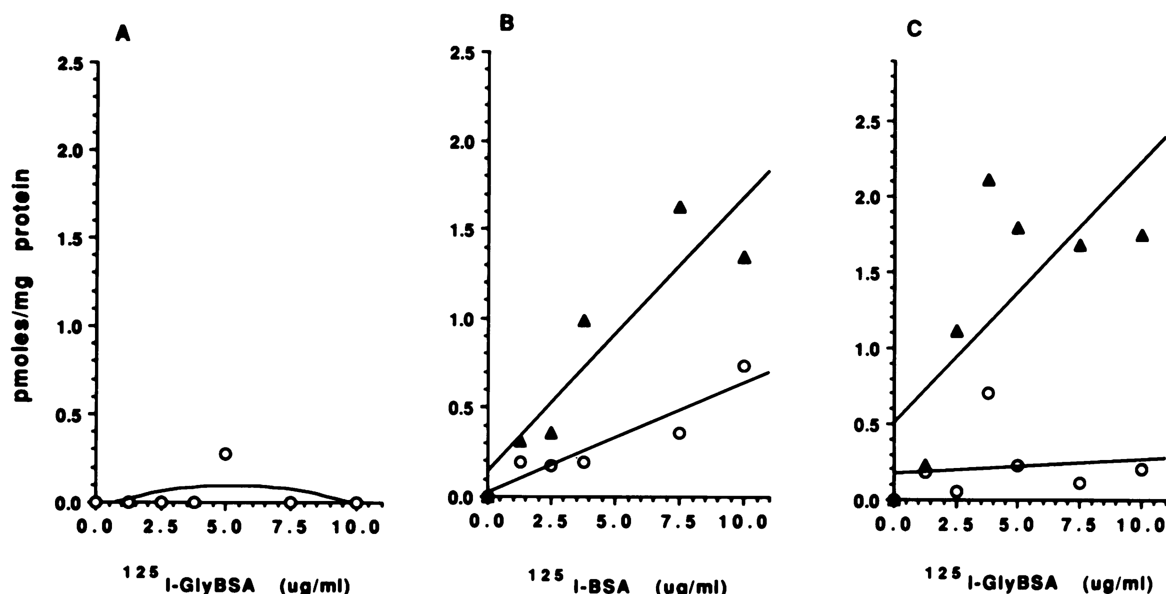

Figure 6. *Specific binding of* ^{125}*I-labeled glycated bovine serum albumin (BSA) to monocytes* (A), *and nonspecific binding of* ^{125}*I-BSA* (B) *and* ^{125}*I-glycated BSA* (C) *to monocytes in the presence of 5 mmol/l (○) or 100 mmol/l (▲) glucose.*

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