Published in final edited form as:
Diabetes. 2006 April ; 55(4): 901–910.

Contribution of Aldose Reductase to Diabetic Hyperproliferation of Vascular Smooth Muscle Cells

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SUMMARY

The objective of this study was to determine whether the polyol pathway enzyme aldose reductase mediates diabetes abnormalities in vascular smooth muscle cell (SMC) growth. Aldose reductase inhibitors (tolrestat or sorbinil) or antisense aldose reductase mRNA prevented hyperproliferation of cultured rat aortic SMCs induced by high glucose. Cell cycle progression in the presence of high glucose was blocked by tolrestat, which induced a G_0-G_1 phase growth arrest. In situ, diabetes increased SMC growth and intimal hyperplasia in balloon-injured carotid arteries of streptozotocin-treated rats, when examined 7 or 14 days after injury. Treatment with tolrestat (15 $mg \cdot kg^{-1} \cdot day^{-1}$) diminished intimal hyperplasia and decreased SMC content of the lesion by 25%. Although tolrestat treatment increased immunoreactivity of the lesion with antibodies raised against protein adducts of the lipid peroxidation product 4-hydroxy *trans*-2-nonenal, no compensatory increase in lesion fibrosis was observed. Collectively, these results suggest that inhibition of aldose reductase prevents glucose-induced stimulation of SMC growth in culture and in situ. Even though inhibition of aldose reductase increases vascular oxidative stress, this approach may be useful in preventing abnormal SMC growth in vessels of diabetic patients.

INTRODUCTION

Diabetes is a major risk factor for the development of cardiovascular disease (1,2). The risk of heart disease among diabetic patients is two- to fourfold higher compared with normal subjects. Diabetes accelerates the progression and increases the severity of atherosclerotic lesions in peripheral, coronary, and cerebral arteries (1). Because both diabetes and cardiovascular disease share a common set of risk factors, it has been suggested that the two diseases share a similar etiology (the “common soil” hypothesis [3,4]). Diabetes also affects cardiovascular responses to injury. It is associated with poor prognosis after myocardial infarction and stroke (1,2). Diabetic patients have a higher propensity for restenosis after percutaneous transluminal coronary angioplasty (5,6), and vascularization does not decrease the excessive mortality rates of diabetic patients (7). Although reasons for the limited efficacy of vascularization remain unclear, occlusive restenosis remains the major determinant of long-term mortality in diabetic patients after coronary balloon angioplasty (6). Even though coronary stenting significantly reduces restenosis, diabetic patients have less favorable clinical outcomes after stent placement (8,9), and diabetes remains a powerful predictor of in-stent restenosis (10,11).

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Diabetes could promote restenosis via multiple mechanisms. Hyperglycemia, hyperinsulinemia, and dyslipidemia could increase inflammation and proliferation in the lesions. Chronic hyperglycemia leads to an increase in the protein kinase C (PKC) activation and accumulation of the advanced glycosylation end products that could dysregulate smooth muscle cell (SMC) growth and mediate retenosis (12). In addition, diabetes also increases the flux of glucose through the polyol pathway (13). Our studies demonstrate that decreasing the polyol pathway activity by inhibiting aldose reductase-mediated conversion of glucose to sorbitol prevents high-glucose-induced diacylglycerol accumulation and PKC activation in SMCs (14). Inhibition of aldose reductase also prevents high-glucose-induced stimulation of the extracellular signal-related kinase/mitogen-activated protein kinase and phosphatidylinositol 3-kinase (15) and activation of nuclear factor-κB (16), thereby decreasing SMC chemotaxis, vascular inflammation, and adhesion. However, the role of aldose reductase in modulating responses to vascular injury remains unclear. We, therefore, determined whether inhibition of aldose reductase prevents high-glucose-induced changes in SMC growth in culture and in injured arteries of diabetic animals. Our data demonstrate that high-glucose-stimulated SMC growth in vitro and diabetic neointimal expansion in injured arteries were both attenuated by aldose reductase inhibition, suggesting that therapies that target aldose reductase may be useful in retarding intimal hyperplasia and restenosis in diabetic vessels.

RESEARCH DESIGN AND METHODS

Penicillin/streptomycin, trypsin, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Life Technologies. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagents used for Western analysis and streptozotocin (STZ) were obtained from Sigma. Lipofectamine was purchased from Invitrogen. Phosphorothioate aldose reductase antisense oligonucleotides were used to transfect vascular SMCs (VSMCs) to prevent the translation of aldose reductase mRNA as described previously (17). Tolrestat and sorbinil were gifts from American Home Products and Pfizer, respectively. Peroxidase-labeled goat anti-rabbit antibodies were purchased from Boehringer Mannheim. Polyclonal antibodies against SMC α-actin and proliferative cell nuclear antigen (PCNA) were from Dako. Polyclonal antibodies against recombinant human aldose reductase were raised in rabbits and characterized as described previously (18). Assay kits for measuring plasma lipids were purchased from Wako Chemicals. Antibodies against protein adducts of 4-hydroxy *trans* 2-nonenal (HNE) were raised and tested using published protocols (19).

Cell culture

SMCs were isolated from rat aorta and maintained in DMEM containing 5.5 mmol/l glucose supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. As described previously (17,18), cell proliferation was determined by trypan blue exclusion, MTT assay, and thymidine incorporation. For counting, cells were trypsinized and counted with a hemocytometer.

Cell cycle distribution

Cell cycle distribution was analyzed by flow cytometry (20). After indicated treatments, the cells were trypsinized, rinsed with PBS, and treated with 20 μg/ml RNase A. DNA was stained with 100 μg/ml propidium iodide and analyzed with a fluorescence-activated cell sorter analysis (FACScan; Becton Dickinson). DNA histograms were analyzed by the ModFit LT V2.0 software (Verity Software House).

Induction of diabetes and tolrestat treatment

Diabetes was induced in adult male Sprague-Dawley rats by STZ (55 mg/kg i.p.) as described previously (16). Control rats were treated with the vehicle only (200 μl 0.05 mol/l sodium citrate, pH 4.5). Blood glucose was monitored after 2 weeks, and only those STZ-injected rats with blood glucose >300 mg/dl were included in the study. Four weeks after treatment, rats were fed tolrestat (15 mg · kg^{-1} · day^{-1}) or vehicle (2.5 mmol/l sodium bicarbonate) by gavage and maintained on rat chow. One day after tolrestat treatment, carotid arteries of 20 nondiabetic and 20 diabetic rats were injured by balloon withdrawal, and these rats were maintained on tolrestat or vehicle for the next 10 and 21 days. Remaining rats were maintained on normal chow for 6 weeks after STZ injection, were fed tolrestat or vehicle for 1 week, and were killed to monitor changes in plasma lipids and tissue sorbitol. Blood was withdrawn by heart puncture in 10 mmol/l EDTA. Plasma lipids were measured by enzyme-linked immunosorbent assay per the manufacturer's instructions. Sorbitol levels in the aorta were measured by gas chromatography (21). Details of the balloon-injury model have been described previously (17,18). All animal protocols were approved by the institutional animal care committee.

Immunohistochemistry and histology

For immunohistochemistry, formalin-fixed specimens of carotid arteries were embedded in paraffin, sectioned at 5 μm, and stained as described previously (18). To measure SMC size, cross-sectional area of the SMC α-actin-positive cells was measured using the Spot Advanced software. Digital images were acquired at ×60 magnification and 500-700 cells from 3-5 consecutive sections from 3-5 rats per group. Because of the wide variations in the size of the proliferative cells closer to the lumen, only 75% of the distal section of the neointima was used to measure SMC. Lesion size was calculated as the ratio of neointima to media using the MetaMorph 4.5 software (Universal Imaging). Collagen and elastin contents in the neointima were visualized by Masson's trichrome staining.

Statistical analysis

Data are expressed as means ± SE and were analyzed by ANOVA and Student-Newman-Keuls tests for multiple comparisons or by Student's *t* test for unpaired data. Statistical significance was accepted at $P < 0.05$ level.

RESULTS

Aldose reductase regulates high-glucose-induced SMC growth in culture

To assess the role of aldose reductase, SMCs cultured in 5.5 mmol/l glucose were growth-arrested for 24 h and then incubated with 5.5 or 25 mmol/l glucose with or without the aldose reductase inhibitors sorbinil and tolrestat. After 24 h, cell growth was determined. Cell cultured in 25 mmol/l glucose grew to nearly twice the level observed with 5.5 mmol/l glucose as determined by MTT assay, by [^{3}H]thymidine incorporation, and by directly counting the number of cells (Fig. 1). More than 80% of the excessive growth in high glucose was prevented by sorbinil and tolrestat. These inhibitors did not affect growth or viability of serum-starved cells incubated with 5.5 mmol/l glucose. Incubation with equimolar concentration of mannitol also did not affect SMC growth (data not shown). These data demonstrate that high glucose increases SMC growth by nonosmotic mechanisms and that treatment with aldose reductase inhibitors prevents high-glucose-induced SMC growth without causing nonspecific loss of cell viability. The effect of inhibitors was further confirmed by using aldose reductase antisense mRNA. As shown in Fig. 2, high glucose stimulated growth of untreated cells and cells treated with lipofectamine or scrambled mRNA but was unable to stimulate the growth of cells transfected with

antisense aldose reductase, in which the expression of aldose reductase was markedly suppressed (Fig. 2*B*). Thus, inhibition of cell growth in high glucose appears to be due to inhibition of aldose reductase and not a nonspecific effect of aldose reductase-inhibiting drugs.

To determine which phase of the cell cycle is affected by inhibiting aldose reductase, we used flow cytometric analysis (Fig. 3). The serum-starved SMCs in 5.5 mmol/l glucose were mostly in the G_0-G_1 stage (81%) with some residual cells in the S (19%) and the G_2-G_1 stages (Table 2). When stimulated with 25 mmol/l glucose, the population of cells in the G_0-G_1 stage decreased to 65% with an increase in the S phase (33%) and the G_2-M phase (2%), indicating that high glucose stimulates cell cycle progression and induces the cells to enter the S phase. Inhibition of aldose reductase prevented the decreased S-phase entry induced by high glucose (from 33 to 21%) and increased the number of cells in the G_0-G_1 phase (78%). Inhibition of aldose reductase did not affect the cell cycle distribution in 5.5 mmol/l glucose. These results suggest that inhibition of aldose reductase prevents high-glucose-induced S-phase entry of SMCs because of a G_0-G_1 block.

Aldose reductase regulates SMC growth in diabetic vessels

We next examined the involvement of aldose reductase in an in vivo model of SMC growth. Using a balloon-injury model of rat carotid arteries, we determined how diabetes affects neointimal expansion of carotid arteries and whether these responses are modified by inhibiting aldose reductase. A total of 40 rats were used in the study; 20 rats were made diabetic, and 20 were nondiabetic. One rat in the nondiabetic group and three rats in the diabetic group died during the study. Their data were not included in the study. All other rats completed the protocol successfully. As shown in Table 1, blood glucose levels were four-to fivefold higher in diabetic than nondiabetic rats. Compared with the nondiabetic group, diabetic rats gained less weight after the induction of diabetes.

We first examined the expression of aldose reductase in normal and injured vessels. In agreement with previous findings with rat (18) and human blood vessels (22), little or no immunoreactivity with anti–aldose reductase antibodies was associated with SMCs in the uninjured arteries of diabetic or nondiabetic rats. In balloon-injured arteries, positive immunoreactivity with anti-aldose reductase antibody was associated with the neointima formed 10 days after injury (Fig. 4). Little or no reactivity was associated with the quiescent cells of the tunica media. Compared with nondiabetic rats, arteries obtained from diabetic rats displayed higher levels of staining with anti-aldose reductase antibody (Fig. 4). After 21 days of injury, higher levels of staining were observed in the nondiabetic vessels; however, there was no significant difference in the extent of staining by the anti-aldose reductase antibody in diabetic and nondiabetic arteries (Fig. 4). Persistence of aldose reductase in the neointima of both diabetic and nondiabetic animals indicates that high levels of aldose reductase are associated with proliferating SMCs in the neointma.

To assess the role of aldose reductase, rats were fed the aldose reductase inhibitor tolrestat. Tolrestat feeding did not affect blood glucose levels (Table 1), although the tolrestat-treated rats gained slightly less weight than the untreated diabetic rats. Diabetic rats displayed increases in plasma cholesterol, phospholipid, and triglycerides. These changes were not affected by tolrestat. Sorbitol concentration in the aorta was much higher in diabetic rats, indicating an increase in aldose reductase activity. Sorbitol concentration was decreased by tolrestat (Table 1), indicating that the drug was effective in inhibiting aldose reductase and in preventing vascular accumulation of sorbitol.

Ten and 21 days after injury, the neointima-to-media ratio was 32-49% higher in diabetic than nondiabetic arteries (Fig. 5). Despite differences in the body weights, no significant

difference was observed in the lumen size of the carotid arteries, suggesting that larger neointima in the diabetic rat was not due to a smaller artery size. The neointima-to-media ratio was significantly lower in the tolrestat-treated nondiabetic and diabetic rats than untreated rats, indicating that inhibition of aldose reductase decreases neointima formation irrespective of diabetes.

To assess SMC growth, arterial sections were stained with anti-PCNA antibodies. The anti-PCNA antibodies stained neointimal cells of both diabetic and nondiabetic animals (Fig. 6). The intensity of staining 10 days after injury was >50% higher ($P < 0.01$) in the diabetic than nondiabetic animals, consistent with a higher proliferative response of diabetic arteries. The staining was more intense in proliferative regions of the lesion. Arterial sections obtained 21 days after injury showed less intense staining than 10-day-old lesions, suggesting that by 21 days, SMC growth had diminished significantly. Treatment with tolrestat significantly ($P < 0.01$) diminished neointimal staining in both diabetic and nondiabetic rats in samples obtained 10 days after injury. However, there was no significant difference in the neointimal staining in the treated and the nontreated groups 21 days after injury (Fig. 6).

The contribution of SMCs to neointima formation was assessed further by staining lesions with anti-SMC α-actin antibodies. Lesions of diabetic rats showed 1.5-fold higher immunoreactivity than controls 10 days after injury (Fig. 7). Treatment with tolrestat diminished this reactivity by 30% in nondiabetic and by 40% in diabetic arteries. In contrast to 10-day-old lesions, 21-day-old lesions of diabetic and nondiabetic rats did not show a difference in their α-actin content. However, tolrestat diminished α-actin in nondiabetic and diabetic rat lesions at 21 days (Fig. 7). These observations suggest that inhibition of aldose reductase prevents SMC growth in the arterial lesions of diabetic rats.

Incubation of SMCs with high glucose has been reported to cause hypertrophy in vitro (23). Therefore, we measured the size of neointimal SMC. The SMC size ranged from 13 to 58 μm^2. The mean SMC size of diabetic lesions did not differ from those of nondiabetic lesions either 10 or 21 days after injury (Fig. 7). Treatment with tolrestat did not affect SMC size in either group (Fig. 7). These observations suggest that at least in the model used, diabetes does not cause SMC hypertrophy and that inhibition of aldose reductase does not affect SMC size in the lesion.

To test whether inhibition of aldose reductase affects the extracellular matrix, we measured the collagen content using the Masson's trichrome stain. Collagen was abundant both in the media and in the neointima. The collagen content in the neointima was higher in the neointima than in the media (Table 2). No significant difference was observed in the collagen levels in the neointima of the diabetic and nondiabetic rats at 10 or 21 days after the injury (Fig. 8). Tolrestat treatment increased collagen levels in the neointima without affecting the media collagen (Fig. 8). Also, high levels of elastin were associated with the media, but these were not affected by tolrestat. The elastin content of the neointima was minimal and was not quantified. Together, these data indicate that although diabetes does not significantly affect collagen levels in the proliferating neointima, inhibition of aldose reductase increases the collagen content of both diabetic and nondiabetic lesions.

Previous studies have shown that balloon-injured arteries display higher levels of protein-HNE adducts (24). In agreement with these findings, we observed that the proliferative regions of the neointima were intensely stained with anti-protein-HNE antibody. No significant difference in staining was observed in 10- or 21-day-old lesions, indicating that protein-HNE adducts are formed during early phases of the injury and then remain unchanged. No statistically significant difference in the extent of protein-HNE adduct

formation was observed between nondiabetic and diabetic lesions 10 or 21 days after injury (Fig. 9), suggesting that during diabetes, contribution of lipid peroxidation to lesion formation is small compared with the oxidative stress generated by the injury itself. However, inhibition of aldose reductase increased the extent of protein-HNE adduct formation (Fig. 9) These data show that even though total neointima was less in the tolrestat-treated group, the residual cells display 1.5- to 1.8-fold stronger immunoreactivity to protein-HNE antibodies. A similar, although somewhat less dramatic, increase in reactivity was observed in diabetic animals treated with tolrestat. The tolrestat-induced increase in protein-HNE adduct formation was apparent in both 10- and 21-day-old lesions, but the effect of tolrestat was not more pronounced in diabetic vessels.

DISCUSSION

The major finding of this study is that inhibition of aldose reductase prevents high-glucose-induced SMC proliferation in culture and that it decreases SMC growth in arterial lesions of diabetic animals. These observations establish a critical role of aldose reductase in mediating SMC growth and suggest that inhibition of aldose reductase may be useful for preventing abnormal SMC growth in diabetic vessels. In most animal models intimal SMC proliferation is the predominant cause of restenosis (25,26), but the effects of diabetes on SMC growth remain unclear. Previous studies on restenosis in diabetic animals have yielded conflicting results. Although previous reports demonstrated increased intimal thickening after balloon injury in alloxan-induced diabetic rabbits (27) and BB Wistar diabetic rats (28), recent studies report exaggerated intimal expansion in obese Zucker rats (29) but not in rats with STZ-induced diabetes (29,30). Coupled with the observation that in some studies, high glucose failed to stimulate SMC proliferation in culture (31,32), it is currently believed that hyperglycemia per se does not stimulate intimal hyperplasia in injured vessels and that insulin resistance or hyperinsulineima is responsible for the increased propensity of human diabetic patients for restenosis. This view is further reinforced by studies (33,34) showing that although rigorous control of hyperglycemia significantly decreases the microvascular complications such as nephropathy and retinopathy, it does not affect macrovascular complications of diabetes. Yet tight glycemic control is associated with lower rate of vessel revascularization in type 2 diabetic patients undergoing percutaneous coronary intervention (35), and it decreases the progression of intima-media thickness in type 1 diabetic patients (36). Moreover, it has been recently reported that elevated HbA_{1c} level is an independent risk factor for coronary artery disease (37). These observations underscore the need for understanding hyperglycemia-specific mechanisms regulating intimal responses to arterial injury. Hence, we examined restenotic changes in STZ-treated rats. In contrast to previous studies in which the short duration (2 weeks) of diabetes may have precluded the effects of high glucose and exaggerated the differences with obese Zucker rats with 8-9 weeks of insulin resistance (29), we induced arterial injury 4 weeks after STZ treatment. With the modified protocol, increased neointimal expansion was observed with diabetes, indicating that hyperglycemia, independent of hyperinsulinemia and insulin resistance, is capable of promoting restenosis.

Several lines of evidence suggest that hyperglycemia exaggerates neointmal formation by promoting SMC growth. Lesions of diabetic rats showed more proliferative activity than those of nondiabetic animals and were stained more intensely with the anti-SMC α-actin antibodies. Because no difference in collagen staining was observed between diabetic and nondiabetic lesions, it appears that the exaggerated neointima formation in diabetic animals could be accounted for entirely by increased SMC proliferation. This is further supported by our data showing that high glucose increases SMC proliferation in culture. Although some previous studies show that high glucose increases SMC proliferation (38,39), in other studies, no mitogenic effects were observed (30-32). Reasons for such discordance are

unclear but may relate to the inability of high glucose to promote growth in the absence of serum or when supra-maximal growth is stimulated by high (5-10%) serum in the growth medium. In our studies, high glucose by itself induced S-phase cell cycle entry of SMC, in the presence of 0.5% serum. The magnitude of the changes in cell cycle was comparable with that reported for rabbit coronary SMCs cultured in 22.2 mmol/l glucose and 1 ng/ml platelet-derived growth factor (40). Thus, our work and that of others suggest that high glucose is a progression rather than a competence growth factor and that it could enhance growth in response to growth factor or cytokine stimulation. Moreover, because cell growth was inhibited by both antisense aldose reductase mRNA and tolrestat, it appears unlikely that inhibition of cell growth in culture is due to nonspecific effect of aldose reductase inhibitors, although non-aldose reductase-dependent effects of the drug in situ cannot be ruled out.

Our data showing that lesion expansion in diabetic animals was diminished by aldose reductase inhibitors further support the in vivo mitogenic effects of high glucose. Our previous work shows that inhibition of aldose reductase prevents SMC growth in culture (18) and in situ in balloon-injured carotid arteries (17). Thus our current data showing that inhibition of aldose reductase prevents high-glucose-induced SMC growth in culture, decreases proliferating cells in the lesion, and decreases its SMC content suggest that aldose reductase in part mediates the proliferative responses of diabetic vessels. In agreement with the cell culture studies, these data also suggest that hyperglycemia does not invoke unique mitogenic pathway but merely exaggerates the effects of other growth factors and cytokines. Thus, agents that prevent SMC growth in normal glucose, peroxisome proliferator-activated receptor γ agonists (20), receptor for advanced glycation end products (41), and aldose reductase inhibitors (this study), also prevent high-glucose–induced SMC growth and arterial lesion formation.

In uninjured arteries, the expression of aldose reductase is largely restricted to the endothelium; however, the enzyme is highly expressed in the proliferating cells, and inhibition of the aldose reductase prevents neointimal hyperplasia (18). Hence, an increase in the expression of aldose reductase in diabetes may be one factor underlying the greater plasticity of diabetic lesions. Our expression studies show that aldose reductase was more abundant in diabetic than nondiabetic arteries. This observation suggests that the exaggerated intimal responses of diabetic animals may stem from their high aldose reductase content. A critical role of aldose reductase in regulating neointima formation is suggested by the data showing a decrease in the neointima-to-media ratio in vessels of rats treated with tolrestat.

In addition to reducing glucose, aldose reductase has also been shown to participate in the detoxification of lipid peroxidation-derived aldehydes such as HNE (13,22,42). Hence inhibition of aldose reductase could increase oxidative stress. Protein adducts of aldehydes accumulate in neointimal lesions in balloon-injured arteries (24), and our previous results show that that HNE is a potent SMC mitogen (43). Hence increased formation of HNE or related electrophiles could be one mechanism for stimulating SMC growth in arterial lesions. However, at high concentrations, these aldehydes are cytotoxic. Although the mechanisms by which aldehydes regulate growth remain unknown, we speculate that aldose reductase controls SMC growth by regulating the abundance and the reactivity of HNE and related aldehydes. Increased aldose reductase activity in arterial lesions maintains these aldehydes in their mitogenic concentration, whereas inhibition of the aldose reductase increases aldehyde concentration and prevents cell growth. This is consistent with our observation that inhibition of aldose reductase was accompanied by a higher accumulation of HNE and decreased lesion progression. Additionally, inhibition of aldose reductase, by promoting HNE accumulation, could inhibit mitogenic signaling pathways activated by

glucose. It has been shown that inhibition of aldose reductase prevents hyperglycemia-induced (14,15,44) and growth factor-induced (17) PKC activation, which is essential for SMC growth in high glucose (45). Regardless of the mechanism, because aldose reductase inhibitors prevent intimal expansion (this study) as well as inflammation associated with nuclear factor-κB activation (16,17,46), without drastically decreasing lesion cellularity (Fig. 10), inhibition of aldose reductase may be a useful strategy not only to decrease lesion size but also to favorably alter lesion composition.

In summary, we have shown that hyperglycemia stimulates rat SMC growth in culture and in situ and that the mitogenic progression effects of glucose are mediated in part by aldose reductase. It has been previously observed that human SMCs in culture (but not in situ [22]) show aldose reductase (18), that aldose reductase expression in human cells is increased during inflammation (22) or growth factor stimulation (18), and that the inhibition of aldose reductase prevents growth of human SMCs (18). In addition, inhibition of aldose reductase also prevents tumor necrosis factor-α-induced expression of adhesion molecules (46) and apoptosis (47) in human endothelial cells. We, therefore speculate that aldose reductase plays a similar role in the development of human lesions as well. If hyperglycemia does increase SMC growth in human lesions, then transient hyperglycemia, especially after restenosis, may be useful in increasing lesion cellularity, which is decreased in diabetes because of impaired adaptive remodeling (48). The paradoxical beneficial effects of hyperglycemia are consistent with the results of the DIGAMI (Diabetes Insulin-Glucose in Acute Myocardial Infarction) study, which demonstrates that acute insulin-glucose infusion after infarction decreases mortality in diabetic patients (49). However, in addition to increasing SMC growth, hyperglycemia could also increase circulatory cytokines (50), which could promote plaque rupture. Hence, anti-inflammatory therapy or concurrent inhibition of aldose reductase by using aldose reductase-inhibiting drugs may be useful in decreasing intimal inflammation and favorably affecting the lesion characteristics in patients with diabetes.

Acknowledgments

S.S. has received National Institutes of Health Grant HL65618. A.B. has received National Institutes of Health Grants ES-11860 and HL59378. S.K.S. has received National Institutes of Health Grant DK36118. K.V.R. has received National Institutes of Health Grant GM-71036.

We are grateful for technical assistance provided by David Young, Debi Greer, and Sharon Lear and the Flow Cytometry and Cell Sorting Core Laboratory at the University of Texas Medical Branch at Galveston.

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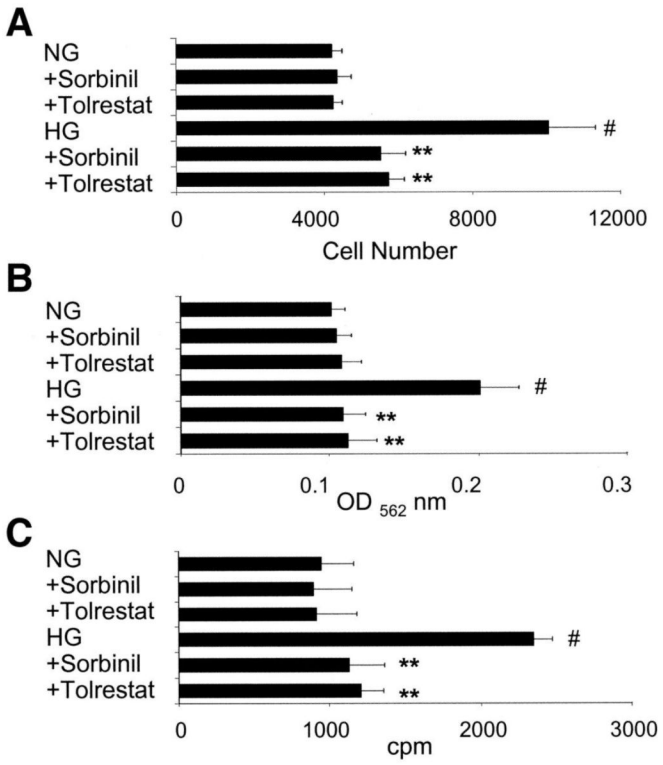

FIG. 1.

Inhibition of aldose reductase prevents high-glucose-induced VSMC growth. Growth-arrested VSMCs in 5.5 mmol/l glucose (NG) were either left untreated or stimulated with additional 19.5 mmol/l glucose (HG) in the absence and presence of 10 μmol/l sorbinil or tolrestat for 24 h. Cell growth was determined by counting the number of cells (*A*), MTT assay (OD_{562}) (*B*), and the incorporation of [^{3}H]thymidine added 6 h before the end of the experiment (*C*). Horizontal bars represent means ± SE ($n = 4$). ** $P < 0.001$ vs. high-glucose cells without the inhibitor; # $P < 0.001$ vs. normal glucose.

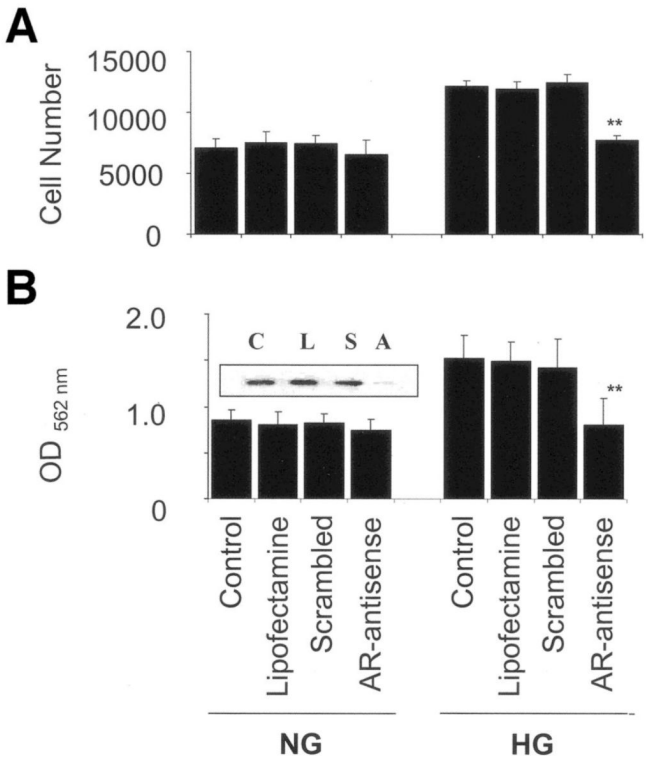

FIG. 2.

Antisense ablation of aldose reductase prevents high-glucose-induced SMC growth. The VSMCs were either left untreated or treated with lipofectamine, aldose reductase (AR) antisense oligonucleotide, or scrambled oligonucleotides and cultured in normal (5.5 mmol/l) glucose (NG) or high (25 mmol/l) glucose (HG) for 24 h. Cell growth measured by counting the number of cells (*A*) and cell viability by MTT assay (OD_{562}) (*B*). The bars represent means ± SE ($n = 4$). *Inset* of *B* shows a representative immunoblot of the control (C), lipofectamine-treated (L), scrambled oligonucleotide–treated (S), and aldose reductase antisense oligonucleotide-treated (A) SMCs probed with anti-aldose reductase antibodies. $^{**}P < 0.001$ vs. cells transfected with the scrambled oligonucleotide.

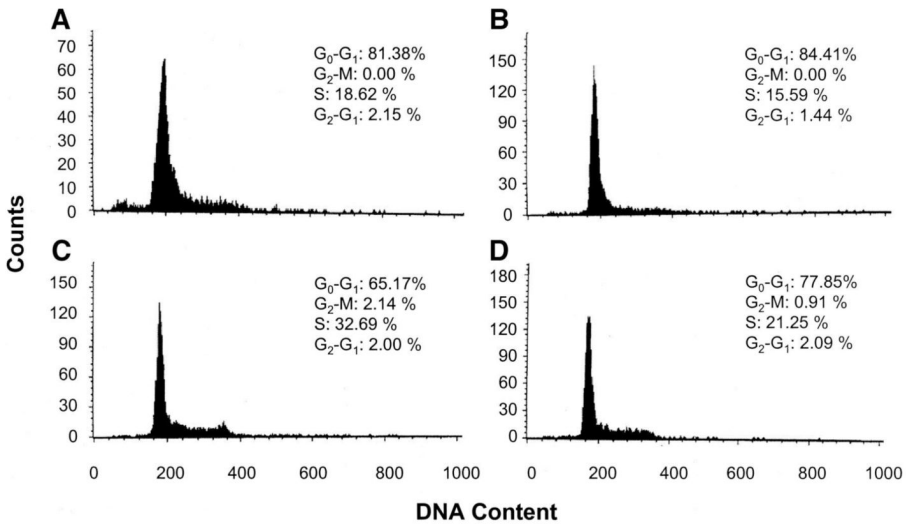

FIG. 3.

Effect of aldose reductase inhibition on high-glucose-induced cell cycle progression. Quiescent VSMCs were incubated without or with sorbinil (10 μmol/l) in DMEM containing 0.1% FBS and 5.5 mmol/l glucose (*A*) with sorbinil (*B*) or 25 mmol/l glucose (*C*) with sorbinil (*D*) for 24 h. The cells were trypsinized, rinsed with PBS, and treated with 20 μg/ml RNase. DNA was stained with propidium iodide, and 1×10^6 cells were analyzed by fluorescence-activated cell sorter analysis. *Inset* shows percent distribution of cells in G_0, G_1, S, G_2, and M stages of the cell cycle for the representative dataset shown in the figure.

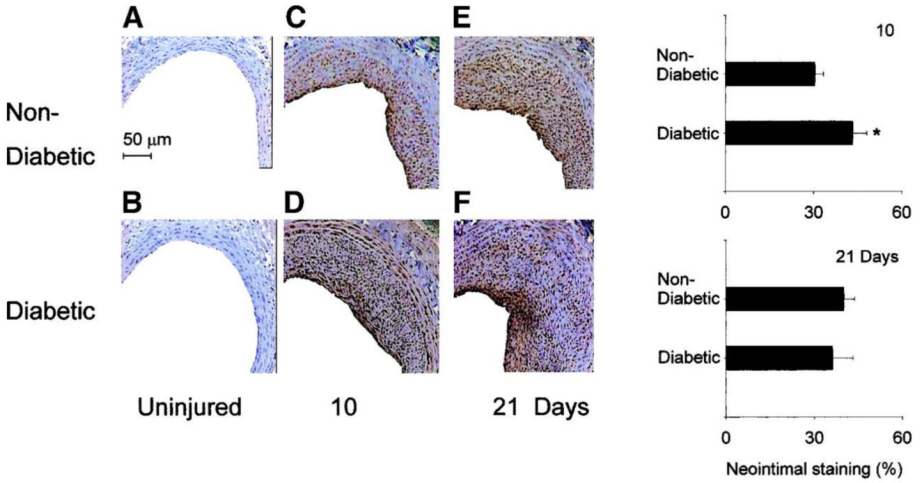

FIG. 4.

Expression of aldose reductase in the proliferating neointima in nondiabetic and diabetic rats. Cross sections of carotid arteries obtained from nondiabetic (control) and diabetic rats without injury (*A* and *B*) or 10 (*C* and *D*) and 21 (*E* and *F*) days after balloon injury were stained with anti-aldose reductase antibody. Immunoreactivity is evident as a dark brown stain, whereas nonreactive areas display only the background color (hematoxylin and eosin staining). The extent of staining was quantified using the MetaMorph imaging software. The bar graphs show means ± SE of neointimal staining (%) 10 and 21 days after injury. **P* < 0.05 vs. control (lesions of nondiabetic rats).

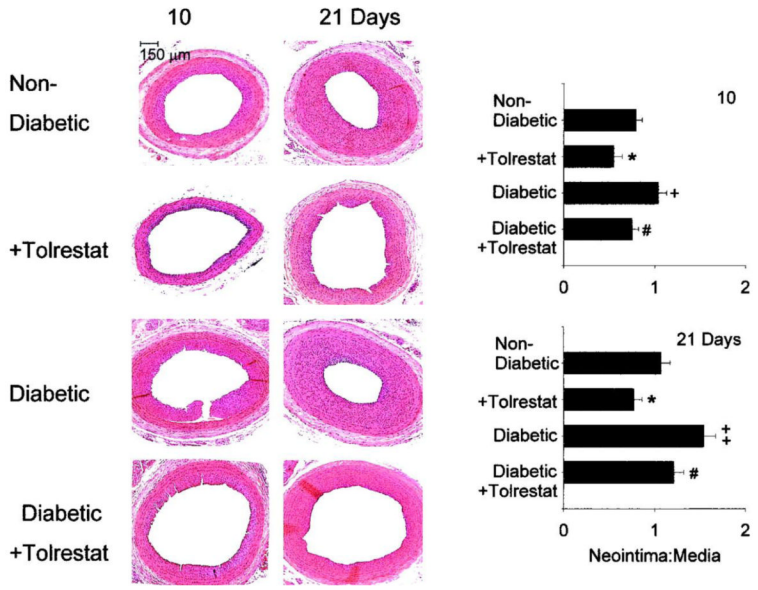

FIG. 5.

Inhibition of aldose reductase diminishes neointima formation in balloon-injured carotid artery of nondiabetic and diabetic rats. Photomicrographs of cross sections of carotid arteries from nondiabetic and diabetic rats at 10 days (*left*) and 21 days (*right*) after balloon injury. Sections were stained with hematoxylin and eosin, and the neointima-to-media ratio was calculated by image analysis. The bar graph shows means ± SE of the neointima-to-media ratios. *$P < 0.05$ vs. control (nondiabetic lesion); +$P < 0.05$ and ++$P < 0.01$ vs. control; #$P < 0.05$ vs. untreated diabetic rats (diabetic).

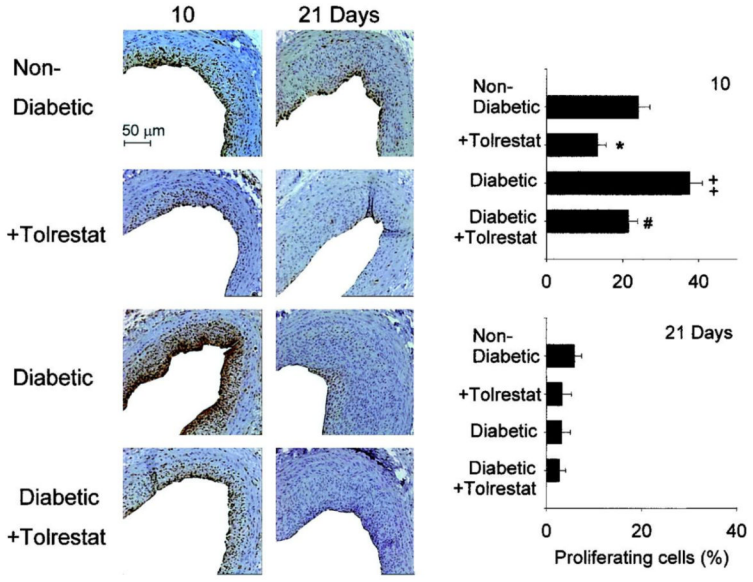

FIG. 6.

Inhibition of aldose reductase prevents cell proliferation in balloon-injured carotid arteries of nondiabetic and diabetic rats. Cross sections of balloon-injured arteries were obtained from nondiabetic and diabetic rats 10 (*left*) and 21 (*right*) days after balloon injury and stained with anti-PCNA. Immunoreactivity is evident as a dark brown stain, whereas nonreactive areas display only the background color. The total number of cells was determined by counting the total number of propidium iodide-positive cells (data not shown), and the number of proliferating cells was determined by counting the number of PCNA-positive cells. The bar graphs show means ± SE. *$P < 0.05$, ++$P < 0.01$ vs. control; #$P < 0.05$ vs. untreated diabetic rats.

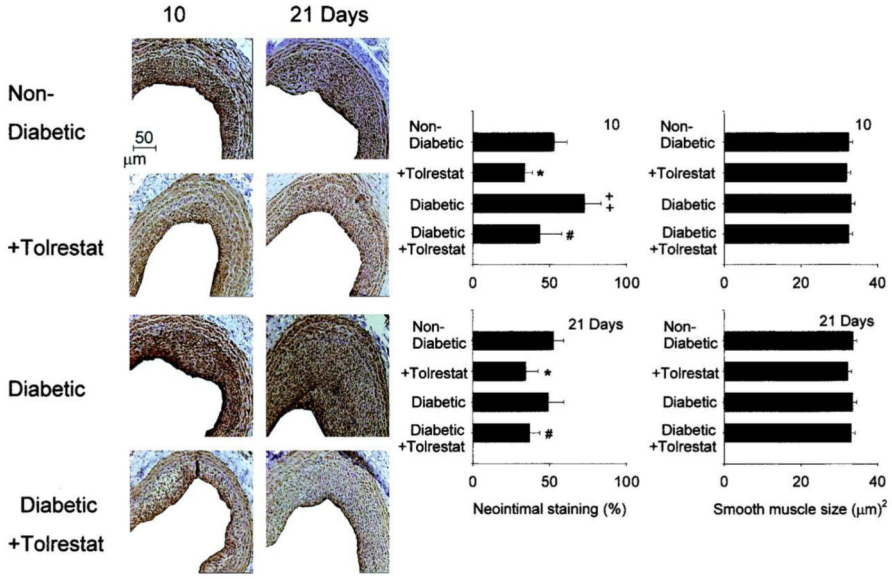

FIG. 7.

Inhibition of aldose reductase diminishes SMC proliferation in the neointima of the balloon-injured carotid arteries of nondiabetic and diabetic rats. Cross sections of balloon-injured arteries were obtained from nondiabetic and diabetic rats at 10 (*left*) and 21 (*right*) days after balloon injury and stained with anti-SMC α-actin antibody. Immunoreactivity is evident as a dark brown stain, whereas nonreactive areas display only the background color. Percent neointimal staining was determined by measuring positive immunoreactivity per unit area. SMCs in the lesion were quantitated by digital image analysis. The bar graphs show means ± SE. *$P < 0.05$, ++$P < 0.01$ vs. control; # $P < 0.05$ vs. sections obtained from untreated diabetic rats.

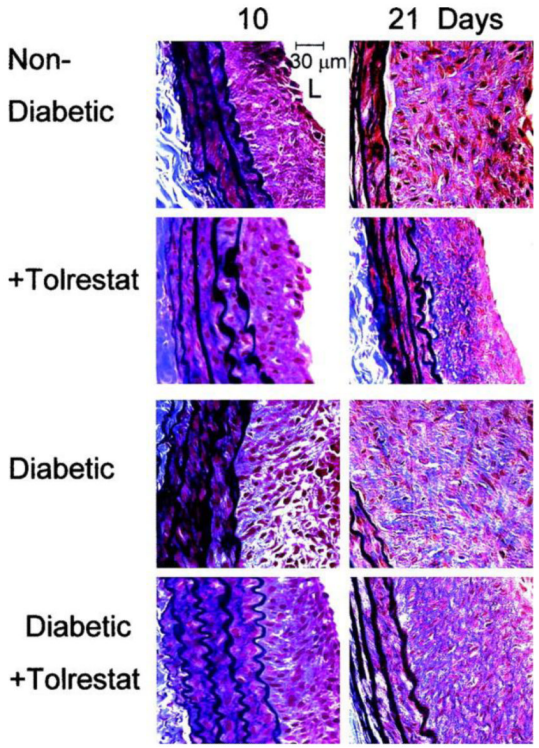

FIG. 8.

Inhibition of aldose reductase increases the abundance of collagen in the neointima of balloon-injured carotid artery of nondiabetic and diabetic rats. Cross sections of carotid arteries from nondiabetic and diabetic rats at 10 (*left*) and 21 (*right*) days after balloon injury, stained with Masson's trichrome. Collagen was stained blue and elastin was stained black. All photomicrographs are in the same orientation. L, luminal surface.

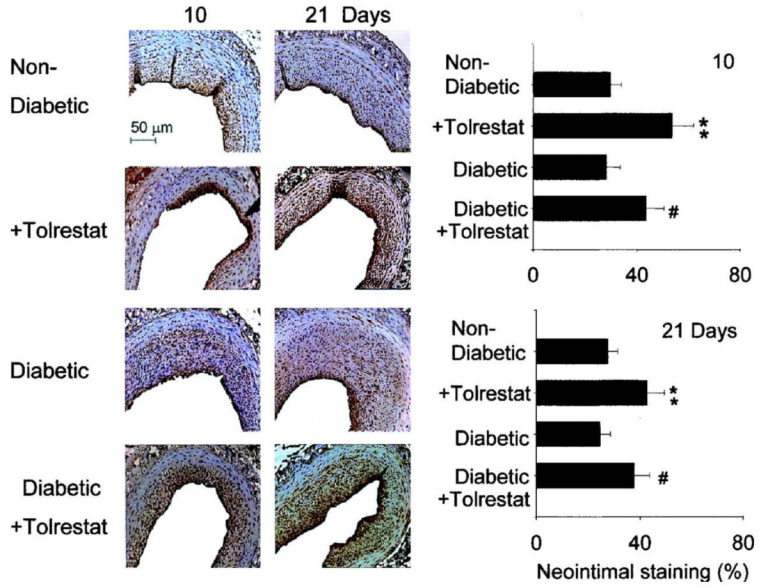

FIG. 9.

Inhibition of aldose reductase increases the abundance of protein-HNE adducts in the neointima of the balloon-injured arteries of nondiabetic and diabetic rats. Cross sections of balloon-injured arteries were obtained from nondiabetic and diabetic rats 10 (*left*) and 21 (*right*) days after balloon injury and were stained with antibody directed against protein-HNE adducts. Immunoreactivity is evident as a dark brown stain; nonreactive areas display only the background color. The bar graphs show means ± SE of the percentage of neointima stained by the anti-protein-HNE antibody. $^{**}P < 0.05$ vs. control; $\#P < 0.05$ vs. untreated diabetic rats (diabetic).

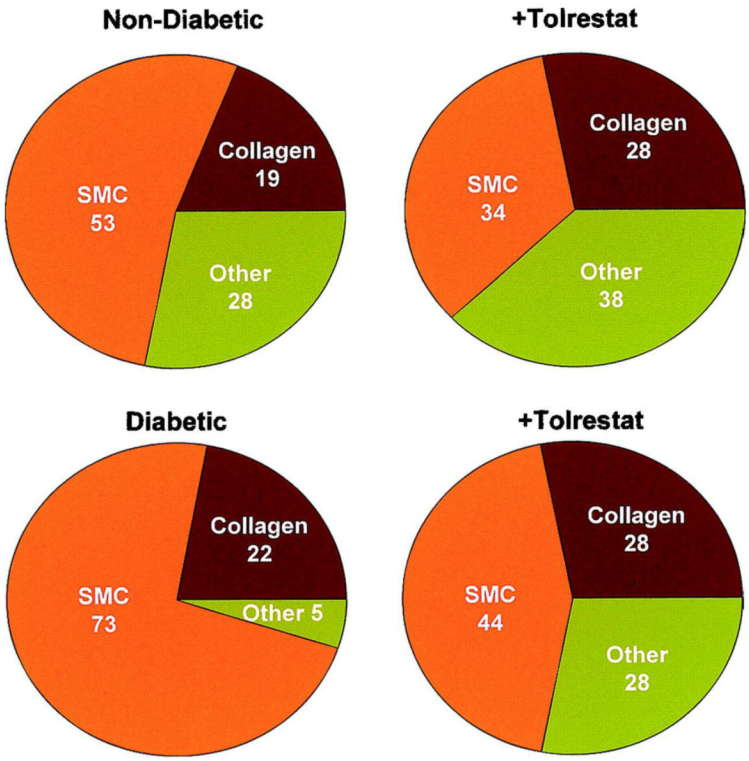

FIG. 10.

Aldose reductase-dependent changes in lesion composition in diabetic and nondiabetic animals. Graph shows percent distribution of SMC, collagen, and other constituents in carotid arteries, 10 days after injury as determined by staining with anti-SMC α-actin and Masson's trichrome stain (see Fig. 7 and Table 2).

TABLE 1

Parameters monitored in diabetic rats

	Nondiabetic	Nondiabetic + tolrestat	Diabetic	Diabetic + tolrestat
Initial body wt (g)	257 ± 6 (8)	267 ± 7 (8)	253 ± 4 (8)	252 ± 4 (8)
Final body wt (g)	381 ± 12 (8)	402 ± 14 (8)	281 ± 15 (8) *	258 ± 11 (8) †
Blood glucose (mg/dl)	116 ± 9 (8)	128 ± 8 (8)	546 ± 48 (8) *	522 ± 41 (8) †
Cholesterol (mg/dl)	68 ± 4 (8)	73 ± 2 (8)	83 ± 5 (8) ‡	88 ± 4 (7) §
Phospholipids (mg/dl)	104 ± 7 (8)	100 ± 2 (8)	145 ± 12 (8) *	160 ± 10 (7) †
Triglycerides (mg/dl)	48 ± 5 (8)	53 ± 5 (8)	215 ± 32 (7) *	199 ± 18 (7) †
Sorbitol (pmol/mg protein)	ND	ND	0.083 ± .011 (5)	0.016 ± 0.003 (6) ¶

Data are means ± SE (*n*). All parameters were determined after 6 weeks of diabetes and 1 week of treatment with tolrestat (15 mg · $kg^{\times 1}$ × day^{-1}) or vehicle (2.5 mmol/l sodium bicarbonate). Sorbitol levels were determined in the aorta by GC-analysis.

* $P < 0.001$ vs. nondiabetic rats;

† $P < 0.001$ vs. nondiabetic rats fed tolrestat;

‡ $P < 0.05$ vs. nondiabetic rats;

§ $P < 0.05$ vs. nondiabetic rats fed tolrestat;

¶ $P < 0.001$ vs. diabetic rats. ND, not detectable.

TABLE 2

Extracellular matrix composition in the balloon-injured arteries

Days after the injury	Nondiabetic + Nondiabetic tolrestat			Diabetic +Diabetic tolrestat	
10	Elastin (media)	24.1 ± 0.3	21.3 ± 3.1	25.5 ± 3	27 ± 3.8
10	Collagen (media)	10.8 ± 1.1	9.8 ± 1.1	10.8 ± 0.9	9.9 ± 1.1
10	Collagen (neointima)	19.2 ± 1.6	27 ± 1.8 *	22.1 ± 2.1	28.1 ± 1.8 †
21	Elastin (media)	28.5 ± 2.3	27.4 ± 5	27.9 ± 6.2	29.1 ± 2.9
21	Collagen (media)	12 ± 0.9	11.6 ± 1.4	12.1 ± 1.4	11.7 ± 1
21	Collagen (neointima)	24.2 ± 1.6	31.6 ± 1 *	25.1 ± 2.9	31.5 ± 1.9 †

Data are means ± SE.

* $P < 0.05$ vs. nondiabetic;

† $P < 0.05$ vs. untreated diabetic rats.