Remodeling of the rat distal colon in diabetes: function and ultrastructure

Marion J. Siegman, Masumi Eto, and Thomas M. Butler

Department of Molecular Physiology and Biophysics, Sidney Kimmel College of Medicine at Thomas Jefferson University, Philadelphia, Pennsylvania

Submitted 4 September 2015; accepted in final form 10 November 2015

Siegman MJ, Eto M, Butler TM. Remodeling of the rat distal colon in diabetes: function and ultrastructure. Am J Physiol Cell Physiol 310: C151–C160, 2016. First published November 11, 2015; doi:10.1152/ajpcell.00253.2015.—This study seeks to define and explain remodeling of the distal colon in the streptozotocin (STZ)-treated rat model of diabetes through analysis of resting and active length dependence of force production, chemical composition, and ultrastructure. Compared with untreated controls, the passive stiffness on extension of the diabetic muscle is high, and active force produced at short muscle lengths is amplified but is limited by an internal resistance to shortening. The latter are accounted for by a significant increase in collagen type 1, with no changes in types 3 and 4. In the diabetic colon, ultrastructural studies show unique, conspicuous pockets of collagen among muscle cells, in addition to a thickened basement membrane and an extracellular space filled with collagen fibers and various fibrils. Measurements of DNA and total protein content revealed that the diabetic colon underwent hypertrophy, along with a proportional increase in actin and myosin contents, with no change in the actin-to-myosin ratio. Active force production per cross-sectional area was not different in the diabetic and normal colon in diabetes, we found remodeling of both smooth muscle cells and the extracellular matrix, characterized by cellular hypertrophy and stiffening, the latter based on increased production of collagen type 1, together with formation of advanced glycation end products (AGES). We show, for the first time, significant alleviation of the stiffness of the diabetic colon through the use of the glycation breaker alagebrium chloride (ALT-711).

SMOOTH MUSCLE forming the wall of hollow organs is notably plastic; that is, it readily adapts to changes in functional demand by remodeling. Changes in functional demand may be driven by changes in neural control or physical factors. For example, the response to obstruction or strain is rapid and results in hypertrophy of the intestine (14), bladder (10), and blood vessels (1, 44), with or without hyperplasia, and this may occur with or without concomitant changes in the extracellular matrix. Functionally, the consequences of such remodeling usually include changes in contractile properties, such as the muscle length dependence of active force production, in the resistance of the resting muscle to extension (passive force and compliance) and the ability to shorten due to structural limits imposed by changes in the relationship between smooth muscle cells and the extracellular matrix through which they interact mechanically (38).

Much has also been learned about the high incidence and changes in motor control of gastrointestinal smooth muscle in disease states, with the focus on autonomic motor and sensory neuropathies (5, 11, 12, 26, 29, 41), but very little is known about changes in the intrinsic mechanical properties of the smooth muscle itself in response to such changes in the control condition. Few, if any, studies have examined the cellular basis of changes in function in response to a change, for example, in neural control, except for Hirschsprung’s disease, which has been analyzed by us in detail and also by others (20, 36, 37).

The metabolic sequelae of diabetes are well known, and complications such as retinopathy, nephropathy, and peripheral vasculopathy have been investigated far more extensively than gastrointestinal motility disorders (reviewed in Ref. 5). Remodeling of the intestinal wall in diabetes, showing marked decreases in compliance, has been documented in vivo in humans and in vitro in animal models (13, 18, 22, 25, 42, 49, 51). These studies, which relied largely on manometric techniques and morphometry of regions of intact intestine in situ or intestinal segments in vitro, defined the stress-strain relationships and provided evidence of increases in tissue weight and thickness of the intestinal wall but provided no direct analysis of cellular mechanisms that could account for the changes in compliance and mechanical function.

In the current study, using the streptozotocin (STZ)-induced model of type 1 diabetes, we focus on the fundamental mechanical properties and ultrastructural relationships that govern force production and compliance of the distal colon of the rat. The STZ model was chosen because of its efficacy in the selective destruction of the insulin-producing beta cells of the pancreas (32) and because it is considered the agent of choice for the production of a reproducible diabetic state (24). The distal colon was chosen because constipation is one of the most common gastrointestinal symptoms among patients with diabetes (5). In diabetes, we found remodeling of both smooth muscle cells and the extracellular matrix, characterized by cellular hypertrophy and stiffening, the latter based on increased production of collagen type 1, together with formation of advanced glycation end products (AGES). We show, for the first time, significant alleviation of the stiffness of the diabetic colon through the use of the glycation breaker alagebrium chloride (ALT-711). Furthermore, in the distal colon in diabetes, ultrastructural evidence demonstrates a unique pattern of redistribution of collagen, consisting of robust linkages joining smooth muscle cells in the extracellular matrix in addition to the formation of pockets rich in collagen fibers scattered among the cells.

METHODS

Animals and Tissues

Production of diabetes. Diabetes was induced by a single injection of the antibiotic STZ (65 mg/kg ip; Sigma-Aldrich, St. Louis, MO) into ~8-wk-old male Sprague-Dawley rats (~300 g body wt; Taconic Biosciences, Hudson, NY) after a 12-h fast. The STZ solution was

Address for reprint requests and other correspondence: M. J. Siegman, Dept. of Molecular Physiology & Biophysics, Thomas Jefferson Univ., 1020 Locust St., Philadelphia, PA 19107 (e-mail: marion.siegman@jefferson.edu).

http://www.ajpcell.org 0363-6143/16 Copyright © 2016 the American Physiological Society C151
Muscles were bathed in a normal Krebs solution [in mM: 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, 1.9 CaCl₂, and 11 glucose (pH 7.4)], which was gassed with 95% O₂-5% CO₂.

DNA and protein measurements. Frozen muscles were weighed and pulverized at liquid nitrogen temperature in 5% trichloroacetic acid for DNA analysis or 2× Laemmli sample buffer (23) for protein analysis. DNA was measured using a modification of the fluorometric method described by Switzer and Sommer (43). Actin and myosin contents were determined by quantitation of SYPRO ruby (Life Technologies)-stained SDS-polyacrylamide gels. The fluorescence of proteins from the muscles was compared with standard curves generated with known amounts of actin and myosin heavy chain run in the same gel as the muscle samples. Approximately 0.8 μg of total muscle protein was added to the gels. Total protein was determined using the Pierce bicinchoninic acid protein assay kit (Life Technologies); bovine serum albumin was used for the standard curves.

Collagen content. Quantitative immunohistochemistry. Muscle strips used for mechanical analysis were fixed at optimum length (L₀) on the myograph in 10% formalin. Generally, four strips from each animal were sampled. The strips were dehydrated in solutions containing increasing concentrations of ethanol and xylene and embedded in paraffin. Subsequently, 5-μm-thick serial sections of each muscle were deparaffinized, rehydrated through graded alcohol solutions, rinsed in deionized water, and subjected to immunohistochemistry. Through the use of serial sections, the distribution of collagen types in each muscle could be assessed and compared. Antigen retrieval was performed in 10 mM citrate buffer, pH 6.0, for 35 min using a steamer. The sections were cooled to room temperature and blocked with 3% H₂O₂ for 15 min and then with 30% goat serum in PBS for 30–60 min. Primary antibodies were incubated for 1 h at 20°C or overnight at 4°C. The following antibodies were used: rabbit anti-collagen-1 (Cell Sciences, Canton, MA; 1:100 dilution), rabbit anti-collagen-3 (Abcam, Cambridge, MA; 1:1,500 dilution), rabbit anti-collagen-4 (Abcam; 1:3,000 dilution), and rabbit anti-AGE (Abcam; 1:1,000 dilution). After the sections were washed in PBS, they were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and then with avidin-biotin-horseradish peroxidase complex (Vector Laboratories) for 30 min. Antibody reactivity was detected using 3,3′-diaminobenzidine (Dako, Carpinteria, CA). Nuclei were counterstained with hematoxylin. To compare the localization of collagen type 1 and AGE, indirect immunofluorescence was conducted using deparaffinized and rehydrated serial sections. No antigen retrieval was performed. The following antibodies were used: rabbit anti-AGE (1:500 dilution) and rabbit anti-collagen-1 (1:20 dilution) with mouse anti-smooth muscle actin (Sigma Aldrich; 1:500 dilution). The primary antibodies and nuclei were visualized using a combination of Cy3-labeled anti-rabbit IgG (Jackson ImmunoResearch; 1:100 dilution) and Cy5-labeled anti-mouse IgG (Jackson ImmunoResearch; 1:100 dilution) with Hoechst 33342 (Sigma-Aldrich; 0.5 μg/ml). Confocal fluorescence imaging was conducted using an Olympus IX70 fluorescence microscope (Plan Apo ×60 oil lens) equipped with a BD Carv II spinning-disk confocal unit. Merged color image processing was conducted using ImageJ software.

Collagen content was quantified by digital image analysis of the sections subjected to immunohistochemistry. For each section, a low-magnification image was acquired using a ×10 objective on a Nikon E600 microscope fitted with a Nikon DS-R1i color camera and run through Nikon Elements D software. Five areas of interest were identified in each section, and images were captured with the ×100 objective using a Qimaging Aqua monochrome camera run through Image-Pro v.7.0 software. Black-and-white images were acquired and imaged through a standard 4′,6-diamidino-2-phenylindole fluorescence filter cube to reduce the blue color and enhance the brown color in the samples. Single images or stacks were acquired at each location. For the latter, Extended Focus in Image-Pro was used, and background images were taken as needed. Each image was opened in Image-Pro, and “auto black objects” count was used to measure total dark area in pixels. The sum of the area for each of the five images was averaged and divided by the total pixel area for an image. The
percentage of the total occupied by the dark pixels for each collagen type from each of the five images analyzed on each section was then determined. The collagen content for each collagen type from all samples was used to calculate the mean ± SE.

BIOCHEMICAL ANALYSIS OF COLLAGEN. Colon smooth muscle strips from rats subjected to the mechanics assay were homogenized (Polytron) with 0.5 ml of PBS. The soluble fraction was removed by centrifugation, and the insoluble residues were weighed. Total collagen in each pellet was extracted overnight with 1 mg/ml pepsin in 0.05 M acetic acid at room temperature, the volume of extraction buffer being adjusted to the wet weight of insoluble residue. The extracts were clarified by centrifugation. The quantitative Sirius Red assay was conducted according to protocol specified in the Sirius Red collagen detection kit (Chondrex, Redmond, WA). A purified total collagen solution (0.1 mg/ml) was used as a standard. Briefly, the insoluble complex of collagen and Sirius Red dye was collected, resolubilized, and then transferred to a 96-well plate. Optical density values at 562 nm of Sirius Red dye precipitated with collagen were determined using a plate reader. For the indirect ELISA, aliquots of the extracts from 0.8 mg of the insoluble residues were absorbed onto 96-well plates. Anti-rat collagen-1 (Cell Sciences; 1:100 dilution), horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch; 1:5,000 dilution), and Super AquaBlue ELISA substrate (eBioscience) were used as primary antibodies, secondary antibody, and a colorimetric reagent, respectively. The mean ± SE was obtained from triplicate assay for each extract. Data from two groups, control (n = 6) and STZ (n = 3), were compared using two-tailed Student’s t-test; P < 0.05 considered to be significant.

Mechanical Behavior: Determination of the Isometric Length-Force Relationship

Each muscle strip was mounted on a myograph consisting of a force transducer (model DSC-6, Kistler-Morse) and a micrometer for adjustment and measurement of length. Signals were recorded on a Gould 220 or RS3200 chart recorder. The muscles were bathed in a flowing Krebs-bicarbonate solution at 37°C, a temperature at which the muscles are devoid of spontaneous mechanical activity. The length-force relationships were determined as described by Gordon and Siegman (17). Muscles were stimulated supramaximally with high-K+ (90 mM) Krebs solution or 5 μM acetylcholine to steady force and then allowed to relax by thorough washout in normal Krebs solutions. After a recovery period of 15 min, the muscle length was increased by 0.5 mm, and following stress relaxation to a steady level of force, the muscle was subjected to a quick release of 0.25 mm before restimulation. The minimum force on release was taken as the passive force for a given length (17, 34). The active force was calculated as the difference between total force produced on stimulation and the passive force at each muscle length. The optimum length for active force production was designated Lo, and the force produced at Lo is Po. Force at any length (P) was normalized to Po, and length (L) was normalized to Lo. Force responses were plotted as P/Lo vs. L/Lo. The minimum length at which passive force is just detectable is Po, as described above, and fixed, while the active force is maximal is designated Lo, and muscle lengths are normalized to Lo. Similarly, active and passive forces are normalized to Po, the maximum active force produced at Lo. The total force is not plotted for the sake of clarity.

Passive force. Passive force in colons from control animals is very low and indistinguishable from that in diabetic colons on extension up to ~0.9 Lo. On further extension, passive force of the diabetic colons increases sharply compared with the controls; the slope of the passive force curve calculated for force at Lo and >Lo for the diabetic colons is 15.5 compared

Ultrastructure

Muscles were set at Lo, as described above, and fixed, while mounted on the myograph, in 2% glutaraldehyde in 0.1 M sodium cacodylate-6% sucrose buffer (pH 7.4). After 15 min the muscles were carefully removed from the myograph and fixed for an additional 105 min. Samples were washed in cacodylate buffer and sequentially treated with 1% osmium tetroxide, washed in buffer, and stained en bloc with uranyl acetate. Then the samples were dehydrated in a graded series of ethanol solutions, infiltrated with propylene oxide followed by graded propylene oxide-Epon solutions, and finally embedded in pure Epon and polymerized overnight at 60°C. Thin sections were stained with lead citrate, examined, and photographed digitally in a Hitachi H7000 electron microscope (3, 35).

RESULTS

Mechanical Characteristics: Length-Force Relationship

The passive and active length-force relationships of circular muscle strips of the distal colon from normal rats and those treated with STZ for 6 wk to create diabetes were determined. In initial trials, muscles were supramaximally activated with acetylcholine and compared with those stimulated with high K+. The results were not distinguishable, but to avoid any changes that might be invoked by differences in signal transduction mechanisms in the two groups of colons, high K+ was used routinely. The length-force relationships are shown in Fig. 1. In Fig. 1, passive force is the resistance to extension of noncontractile components of the muscle, and active force results from the interaction of myosin cross bridges with actin filaments on stimulation at a given muscle length. The muscle length at which active force is maximal is designated Lo, and muscle lengths are normalized to Lo. Similarly, active and passive forces are normalized to Po, the maximum active force produced at Lo. The total force is not plotted for the sake of clarity.

Passive force. Passive force in colons from control animals is very low and indistinguishable from that in diabetic colons on extension up to ~0.9 Lo. On further extension, passive force of the diabetic colons increases sharply compared with the controls; the slope of the passive force curve calculated for force at Lo and >Lo for the diabetic colons is 15.5 compared

Fig. 1. Length-force relationships in strips of circular muscle of distal colon from normal and streptozotocin (STZ)-treated diabetic rats. Muscles were stimulated with high-K+ Krebs solution. Passive force (●) and active force (▲) of strips from normal and STZ-treated muscles (~ and –, respectively) are plotted as a function of muscle length (L) relative to optimum length (Lo). Force responses (PPo) are normalized to maximum active force produced at Lo (Po). Values are means ± SE of 2–4 muscle strips from each of 12 normal and 12 STZ-treated animals.
with 8.1 for controls ($P \leq 0.0001$, $n = 12$ animals for each condition).

**Active force.** There is both an amplification of force and a broadening of the range of muscle lengths over which active force can be produced in the diabetic colons. In contrast to the range of muscle length in controls, from $\sim 0.42 \, L_o$ to $L_o$, the range of lengths extends from $\sim 0.2 \, L_o$ to $L_o$ in the diabetic group. The average force per cross-sectional area (calculated from $P_o$ and muscle dimensions at $L_o$) was not significantly different in the control and STZ-treated muscles ($0.301 \pm 0.059$ and $0.501 \pm 0.103$ N/mm$^2$, $P = 0.2$, $n = 12$ animals for each condition).

**Collagen, DNA, Total Protein, and Contractile Proteins**

The distribution of collagen types 1, 3, and 4 in the muscle strips was determined to learn whether the relative concentrations of these scleroproteins were changed in the diabetic colons and how they correlated with the observed changes in length-force relationships. The collagen content of colon samples used for mechanics, in addition to that of adjacent segments, was determined by two methods: quantitative immunohistochemistry and biochemical analysis. The results are shown in Figs. 2 and 3. On the basis of quantitative immunohistochemical analysis of serial sections of the preparations, the colons from diabetic animals showed a change in the distribution of collagen types. Specifically, there was a doubling of collagen type 1 in the diabetic colons ($P < 0.05$, $n = 25$ for each condition) but no significant changes in the relative amounts of collagen type 3 or 4. There was also a significant increase in AGEs in the STZ-treated animals ($P < 0.05$, $n = 25$; Fig. 2). Biochemical analysis of samples revealed a significant increase in collagen type 1 and a significant decrease in collagen type 3 in the diabetic colons but no significant differences in total collagen (Fig. 3). The discrepancy in these results for collagen type 3 and those from quantitative immunohistochemistry may be attributed to the uncertainties of complete extraction of the residues from samples prepared for chemical analysis. However, the increase in collagen type 1 in these samples is consistent with and predicted by the stiffening of the distal colon of diabetic animals, as shown in Fig. 1.

The DNA content of the colons of diabetic animals decreased significantly after 6 wk of treatment with STZ compared with untreated animals (Fig. 4); however, the total protein content of the same samples were not different. The actin and myosin contents of the muscles were also measured. The concentration of contractile proteins of control and diabetic colons, on the basis of wet weight of tissue, did not differ significantly, nor did the actin-to-myosin ratio (Fig. 5). The decrease in DNA per wet weight is consistent with cellular hypertrophy, but the lack of change in the myosin and actin contents per gram wet weight indicates that these proteins increase in proportion to the increase in total muscle mass. The myosin content should determine the number of cross bridges available to develop force through their interaction with actin.

![Fig. 2. Quantitative immunohistochemical analysis of collagen content of strips of circular muscle of distal colon from normal and STZ-treated diabetic rats. Serial sections of strips used in mechanics experiments were labeled for collagen types 1 (Col1), 3 (Col3), and 4 (Col4) and advanced glycation end products (AGEs). Values are means ± SE of 25 for each condition.](http://ajpcell.physiology.org/)

![Fig. 3. Collagen expression in distal colon smooth muscle of normal and STZ-treated diabetic rats. Total collagen was quantitatively extracted from insoluble residues of smooth muscle tissues. A: extent of collagen types 1 and 3 in tissues from normal animals was set as 1, and relative value (Relative COL) is shown. Extent of total collagen ($\mu$g/mg of insoluble residues) was obtained using purified collagen preparation as standard. Values are means ± SE; $n = 3–6$. *$P < 0.05$. B: total collagen (Total COL) in distal colon from control, untreated animals and animals treated with STZ analyzed in triplicate. Values are means ± SE of 6 animals for each condition.](http://ajpcell.physiology.org/)

![Fig. 4. DNA and total protein content of distal colon smooth muscle from normal and STZ-treated diabetic rats. Values are means ± SE of 6 animals for each condition. *$P < 0.05$.](http://ajpcell.physiology.org/)
The similarity of myosin content in the diabetic and control animals is consistent with the similarity of the active force produced per cross-sectional area. Taken together, these results indicate that, in diabetes, there is a hypertrophy with a proportional increase in contractile proteins and that collagen type 1 constitutes a greater fraction of the total protein.

**Role of AGEs in Colonic Stiffening**

The formation of advanced AGEs that cross-link collagen fibrils in the etiology of diabetic complications is well known (4), and it was of interest to determine the extent to which the production of AGEs contributed to the observed stiffening of the colon. Our strategy was to assess the effect of the glycation "breaker" ALT-711 on mechanical behavior of the distal colon. Two groups of animals (n = 6 for each condition) were studied: one was treated with STZ for 5 wk and subsequently treated with ALT-711 for an additional 5 wk, and the other was treated with STZ alone for 10 wk. The rationale for this regimen was based on results showing that the DNA contents of the colons from STZ-treated animals were similar at 6 and 10 wk (0.35 ± 0.04 and 0.42 ± 0.07 μg/mg wet wt, P = 0.29, n = 6), as were the total protein contents (40.69 ± 7.34 and 51.87 ± 9.39 mg/g wet wt, P = 0.33, n = 6), indicating that hypertrophy of smooth muscle had reached a steady state. We also assumed that by 5 wk the stiffness of the colon would have increased sufficiently for the effect, if any, of ALT-711 to be discerned in mechanics experiments. In the STZ- and STZ + ALT-711-treated animals, blood glucose levels (345 ± 36 and 366.0 ± 71 mg/dl, respectively, P = 0.71, n = 6) and body weights (237 ± 54 and 254 ± 54 g, respectively, P = 0.78, n = 6) were also not significantly different after the 10-wk treatment period. In limited assays, the relative contents of collagen types 1, 3, and 4 after 10 wk of treatment with STZ (n = 3 animals, 5 assays each) were similar to those of animals that were treated for only 6 wk (data shown in Fig. 2). Also, in this trial the AGE contents after 10 wk were 5% higher than in untreated controls (data shown in Fig. 2) but similar to collagen type 1, 3, and 4 contents (P > 0.05, n = 3 animals, 5 assays each).

The mechanical properties of the colons from the two groups of animals were measured, and the resulting length-force relationships are shown in Fig. 6. There was no significant difference in the resting compliance of the muscles within the range of 0.65 \( L_o \) to \( L_o \), suggesting no significant effect on possible glycation of elastin. However, there was a significant increase in the resting compliance of the diabetic colon of animals treated with ALT-711 when tissue length was extended beyond \( L_o \); the slope of the passive length-force relationship as calculated from force at \( L_o \) to 1.2 \( L_o \) decreased significantly (2.94 vs. 1.18, \( P \leq 0.0001 \)). Also, in contrast to the constancy of active force production at short lengths in the range 0.8 \( L/L_o \) to 0.7 \( L/L_o \) of the diabetic colons, active force continued to decline at \( L < 0.8 L/L_o \) in the ALT-711-treated colons. These results suggest that an internal resistance to shortening and force production was reduced as a result of treatment with the glycation breaker.

**Ultrastructure**

Examination of tissue sections prepared for immunohistochemistry revealed unusual dense patches of collagen fibers and elastic elements among the smooth muscle cells in the diabetic colons (Fig. 7B) that did not occur in controls (Fig. 7A). In Fig. 7, the colon preparations were stained for AGEs, but similar results were obtained for collagen types 1, 2, and 4. Furthermore, staining of serial sections showed that the distribution of AGEs coincided with that of collagen type 1 in the patches (Fig. 7, C and D). These findings prompted the examination of samples using electron microscopy.

Smooth muscle in colon samples from untreated animals (Fig. 8) is quite similar to that in other gastrointestinal muscle preparations (38); contractile filaments run parallel to the longitudinal axis of the cells, there are abundant dense bodies within the cytoplasm, and at the plasma membrane, regions rich in caveolae are interspersed among regions with dense bodies. The extracellular space shows abundant collagen fibers and other fibrous materials linking neighboring smooth muscle cells. Higher-magnification views (Fig. 8, B and C) reveal details of regions in which the plasma membranes of adjacent cells are in close apposition, attached by fine fibrous elements in the extracellular space. These regions differ from the “intermediate junctions” originally described by Gabella (15), which form a true mechanical junction between cells. Collagen fibers, identified by their characteristic striation pattern, are linked to the plasma membrane at the mouth of caveolae and plasma membrane (Fig. 8).
Within the cytoplasm, actin filaments can be seen attached to the plasma membrane dense bodies. The diabetic colon (Fig. 9) has numerous distinguishing features, including a sharply delineated basement membrane, plasma membrane dense bodies extending over long regions of the plasma membrane, an extracellular space filled with fibrous material interconnecting neighboring muscle cells, and conspicuous patches containing collagen fibers (see also Fig. 7B). At higher magnification, these patches (Fig. 9B) show collagen fibers together with microfibrils, possibly fibrillin, attaching adjoining cells. These occur at dense body and caveolar regions. Structural characteristics are similar in preparations from animals treated with ALT-711 (Fig. 9, C and D). The complexity and variability of the patches in the diabetic colons cannot be overstated and depend largely on the orientation of the cells and level of each section, reflecting to a considerable extent the configuration of collagen fibers and other fibrils in the extracellular space, often in a twisted configuration.
DISCUSSION

The overall goal of this investigation was to determine the cellular basis of mechanical properties of intestinal smooth muscle in diabetes. We were interested in the distal colon because diabetic patients experience constipation and the malfunction of this region would be a contributing, if not causal, factor. With the circular muscle of the distal colon of the STZ-treated rat used as an experimental model, the major mechanical findings were a marked decrease in resting compliance, together with a small, but significant, amplification of force production at short muscle lengths. The stiffening of smooth muscle of the distal colon was shown to be based on an increased production of collagen type 1 and AGEs. The distribution of fibrotic elements of the extracellular matrix includes an increase in density of matrix joining adjacent smooth muscle cells, as well as dense patches of collagen fibers and fibrils among the muscle cells. One can only speculate that these structures reinforce the extracellular matrix of the diabetic colon. In addition, there was thickening of the basement membrane, suggesting that adhesion sites may be

Fig. 9. Electron micrographs of longitudinal sections of circular muscle of STZ-treated (A and B) and STZ + ALT-711-treated (C and D) rat distal colon. A: STZ alone. Dense bodies extend long distances along the plasma membrane of smooth muscle cells. Extracellular space separating smooth muscle cells is tightly packed with extracellular matrix elements (white arrowheads). Collagen-rich patch is indicated by arrow. B: high-magnification view of a collagen patch. Black arrow points to a collagen-rich patch, which is filled with many fibers and fibrils (white arrowheads). Plasma membrane is very dense in this region and in a neighboring cell (black arrowheads). C: STZ + ALT-711. Dense bodies are abundant in the cytoplasm. Extracellular space separating neighboring smooth muscle cells is slender, and the basement membrane is thickened (black arrowhead). Caveolae are abundant along the plasma membrane. Collagen patches are conspicuous and are filled with amorphous collagenous elements (arrow) as well as collagen fibers (open arrowhead). D: STZ + ALT 711. High-magnification view of a collagen patch shows thickened basement membrane (black arrowhead) and clusters of caveolae (arrow). Collagen fibers (clear arrowhead) in various orientations, together with fine fibrils, likely fibrillin (open arrowhead), fill the patch.
modified in fibrosis and occur when high resistive forces are generated. Thickening of the basement membrane has also been reported for the small intestine (33). Clearly, extensive remodeling of the extracellular matrix has occurred in the diabetic distal colon. The functional consequences of such remodeling, based on extensive examination of normal tissues that are similarly rich in collagen (38), are complex. First, stiffening of the extracellular matrix impedes distension and filling of the colon. Second, stiffening of the extracellular matrix impedes shortening and emptying by providing a resistance against which cross bridges must pull on stimulation. Third, compression of the stiff matrix during shortening results in smooth muscle cell and filament misalignment and dissipation of force, together with compromise of cellular mechanotransduction mechanisms.

The remodeling of the extracellular matrix of the distal colon occurs together with hypertrophy of smooth muscle, with a proportional increase in the contractile proteins actin and myosin per gram wet weight and no change in the actin-to-myosin ratio. Functionally, the latter results are consistent with the lack of change in active force production per cross-sectional area. These results suggest that the contractile machinery undergoes remodeling when high resistive forces of the extracellular matrix are generated. Future studies are required to determine the signals that initiated the remodeling, together with a definition of the extent to which plasma membrane adhesion sites and the cytoskeleton have been modified to balance the outside-in and inside-out forces that are generated in the intact tissue.

The most significant components of visceral and vascular smooth muscles that underlie their passive mechanical properties are collagen and elastin. These elements are essential for conduit and reservoir function by providing a resistance on extension and preventing overdistension on filling; the elastic recoil of these elements facilitates propulsion of contents. The most rigorous analyses of extracellular matrix components have been done on vascular smooth muscles. From these studies, it is widely accepted that, at short muscle lengths (low values of strain), the low resistive force on extension is due to the extensibility of elastin. The major part of resistance on further extension is due to the recruitment of collagen fibers, which are considerably stiffer than elastin (8, 9, 40, 52). The collagens are genetically distinct macromolecules. Collagen type I has substantial tensile strength, whereas collagen type 3 is more resilient and supports the structural integrity and distensibility of the extracellular matrix. Collagen type 4 and laminin are characteristic basement membrane components, and collagen type 5 and the adhesion protein fibronectin occur in basement membranes and the interstitium (46).

In the extracellular matrix, elastin, collagen, and laminin are subject to biochemical changes that can affect their mechanical properties. There is abundant evidence that the production of AGEs through the nonenzymatic glycation and oxidation of proteins, lipids, and nucleic acids results in the cross-linking of extracellular matrix proteins. As a result, production of AGEs may contribute to the complications of diabetes, in addition to the progression of cardiovascular disease and aging (4, 19, 21). It is the chronic hyperglycemia in diabetes that increases the generation of AGEs. High levels of glucose nonenzymatically react with long-lived proteins, such as collagen, forming reversible Schiff base intermediates and Amadori products. The Amadori structure undergoes additional chemical alterations to be eventually converted to stable cross-linked AGEs (45). AGEs also accumulate under normoglycemic conditions, where the hydrolytic turnover rate of collagen is diminished, as in aging, resulting in the increased stiffness and content of collagen. AGEs also increase the expression of collagen types. In both aging and diabetes, expression of the AGE receptor RAGE increases (50). Functionally, these changes are of particular relevance in smooth muscles, in which the mechanical interaction among muscle cells in tissues occurs through the extracellular connective tissue matrix to which they attach. The extracellular matrix not only is important in force transmission and cell shortening, but because of its ability to undergo remodeling, it is also important in the control of tissue homeostasis (1, 28, 37, 44). Taken together, the AGE/RAGE cascade and increased matrix accumulation appear to contribute to the loss of distensibility of the arterial wall and myocardial stiffening of diabetes and aging (2, 27, 31). How the AGE/RAGE cascade initiates these functional changes is of great interest and a growing area of investigations (30, 39) that has been reviewed in great detail by Ott and colleagues (30) and by Zhao and colleagues for the diabetic heart (50).

It is well known that the complications of diabetes extend also to the gastrointestinal tract. In rodent models of diabetes (STZ), the stiffness of the esophagus, small intestine, and proximal colon, based primarily on analyses of stress-strain relationships, has been shown to increase (13, 16, 22, 49, 51). The increased stiffness of the various regions studied was associated with an increase in total collagen content (42). Remodeling of the extracellular matrix in diabetes also extends to proteins such as laminin-1 and fibronectin of the smooth muscle layer of the small intestine, which increase in concert with a thickening of the basement membrane. These effects, which could be reversed by treatment with insulin, support the view that hyperglycemia may mediate the remodeling (33). Furthermore, the upregulated expression of AGEs in diabetic rat models has been demonstrated in the villi of the jejunum, ileum, and proximal colon, as well as in smooth muscle (7, 48).

In the current study, diabetic rats were treated with ALT-711, a glycation breaker. The use of ALT-711 to break existing glucose-derived cross-links provided a direct means of assessing functionally the contribution of cross-linking to the diabetes-associated change in stiffness (45). We found that treatment with the glycation breaker ALT-711 increased the resting compliance of the STZ colon but did not change the collagen distribution of the muscle. Furthermore, the treatment extended the range of muscle lengths over which active force and shortening could occur, reflecting the removal of the resistance to shortening from an otherwise stiff extracellular matrix. Fundamentally, no differences between the structure of colons from STZ-treated and STZ + ALT-711-treated animals could be discerned. Certainly, one would not expect glycated linkages to be electron-dense and, thereby, visible in electron micrographs. In the face of similarities in the distribution of collagen types in these tissues, one can only conclude that ALT-711 increased the compliance of the colon strictly by breaking glycated links among existing collagen fibers in the remodeled muscle.

These results are, to our knowledge, the first to show the ability of an AGE breaker to alleviate colonic stiffening and its adverse functional consequences in an in vivo model of diabetes. The absence of change in collagen distribution following
treatment with the glycation breaker is consistent with the findings from a study of the STZ-treated canine heart by Candido et al. (6) but contrary to results from study of an alloxan model of diabetes by Liu et al. (27), where ALT-711 appeared to decrease collagen content in large arteries. With the establishment of this model, the way is now open to future studies that will investigate the mechanisms underlying the remodeling of the distal colon and, hopefully, identify productive therapeutic interventions.

ACKNOWLEDGMENTS

We gratefully acknowledge the contribution of James Hayden (Imaging Shared Resource of the Wistar Institute) for devising and performing the quantitative analysis of samples subjected to immunohistochemistry. We are also indebted to Prof. Andrzej Fertala (Rothman Institute at Thomas Jefferson University) for advice on the chemical assays of collagen and to Susan U. Mooers and Diana Menezes for technical assistance.

GRANTS

This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-09143201 (M. J. Siegman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.J.S. developed the concept and designed the research; M.J.S., M.E., and T.M.B. performed the experiments; M.J.S., M.E., and T.M.B. analyzed the data; M.J.S., M.E., and T.M.B. interpreted the results of the experiments; M.J.S., M.E., and T.M.B. prepared the figures; M.J.S. and T.M.B. drafted the manuscript; M.J.S., M.E., and T.M.B. edited and revised the manuscript; M.J.S., M.E., and T.M.B. approved the final version of the manuscript.

REFERENCES