Original Paper

Advanced Glycation End Products Impair Ca^{2+} Mobilization and Sensitization in Colonic Smooth Muscle Cells via the CAMP/PKA Pathway

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Key Words

Diabetes • Colonic dysmotility • AGEs • RAGE • CAMP/PKA pathway

Abstract

Background/Aims: Excessive production of advanced glycation end products (AGEs) has been implicated in diabetes-related complications. This study aimed to investigate the mechanism by which AGEs potentially contribute to diabetes-associated colonic dysmotility. **Methods:** Control and streptozotocin (STZ)-induced diabetic groups were treated with aminoguanidine (AG). The colonic transit time and contractility of circular muscle strips was measured. ELISA, immunohistochemistry and western blotting were used to measure *N*^ε-carboxymethyl-lysine (CML) levels. Primary cultured colonic smooth muscle cells (SMCs) were used in complementary *in vitro* studies. **Results:** Diabetic rats showed prolonged colonic transit time, weak contractility of colonic smooth muscle strips, and elevated levels of AGEs in the serum and colon tissues. cAMP levels, protein kinase-A (PKA) activities, and inositol 1,4,5-trisphosphate receptor type 3 (IP₃R3) phosphorylation were increased in the colon muscle tissues of diabetic rats, whereas RhoA/Rho kinase activity and myosin phosphatase target subunit 1 (MYPT1) phosphorylation were reduced. The inhibition of the production of AGEs (AG treatment) reduced these effects. In cultured colonic SMCs, AGE-BSA treatment increased IP₃R3 phosphorylation and reduced intracellular Ca^{2+} concentration, myosin light chain (MLC) phosphorylation, RhoA/Rho kinase activity, and MYPT1 phosphorylation. The PKA inhibitor H-89 and anti-RAGE antibody inhibited the AGE-BSA-induced impairment of Ca^{2+} signaling and cAMP/PKA activation. **Conclusion:** AGEs/RAGE participate in diabetes-associated colonic dysmotility by interfering with Ca^{2+} signaling in colonic SMCs through targeting IP₃R3-mediated Ca^{2+} mobilization and RhoA/Rho kinase-mediated Ca^{2+} sensitization via the cAMP/PKA pathway.

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Introduction

Gastrointestinal (GI) tract dysmotility is common in patients with diabetes mellitus (DM) and the GI symptoms of diabetes are gastroparesis, diarrhea, constipation, and fecal incontinence [1]. Community surveys reported that constipation is the most common GI symptom seen in 60% of the patients with DM [2, 3]. Diabetic complications, particularly peripheral neuropathy and poor glycemic control, are the independent risk factors for persistent GI symptoms in patients with diabetes [4]. GI smooth muscle (SM) dysfunction is considered one of the causes for these GI complications, although other factors including autonomic neuropathy, loss of enteric neurons, and decreased number of interstitial cells of Cajal are also involved [5–7]. The increased formation of AGEs is generally regarded as one of the main mechanisms responsible for diabetes-related complications [8]. AGEs are produced by post-translational modification of proteins via non-enzymatic glycation, and their formation can be monitored *in vivo* with *N*^ε-carboxymethyl-lysine (CML), a biomarker of AGEs [9]. Previous studies have shown that contractility of the ileum in rats with diabetes in response to flow and ramp distension is associated with the expression of AGEs and the receptor for advanced glycated end products (RAGE) [10]. In addition, Chen et al. reported that muscle layers in the small intestine and colon of diabetic rats showed positive immunostaining for AGEs/RAGE [11]. However, the precise role of AGEs in diabetic GI SM dysfunction is unclear.

In the mammalian GI tract, the contraction of SMCs is initiated by extracellular Ca²⁺ entrance through L-type Ca²⁺ channels and by intracellular Ca²⁺ release from inositol 1, 4,5-trisphosphate receptor 3 (IP₃R3)-operated Ca²⁺ stores of endoplasmic reticulum [12]. This is referred to as Ca²⁺ mobilization, which results in the activation of myosin light chain (MLC) kinase (MLCK) and transient MLC20 phosphorylation and contraction [12]. In contrast, sustained contraction reflects the activation of the monomeric G protein RhoA, resulting in the inhibition of MLC phosphatase (MLCP) via Rho kinase-mediated phosphorylation of myosin phosphatase target subunit protein-1 (MYPT1), a regulatory subunit of MLCP, and PKC-mediated phosphorylation of CPI-17, an endogenous inhibitor of MLCP. The inhibition of MLCP activity leads to sustained MLC phosphorylation and contraction, and is referred to as Ca²⁺ sensitization [12]. In streptozotocin (STZ)-induced diabetic rats, a decreased contractile response of colon SM has been reported to result from altered intracellular Ca²⁺ mobilization [13]. Diabetic mice also showed altered expression and phosphorylation of Ca²⁺ sensitization proteins in gastric antrum SM [14]. Nevertheless, the mechanisms of impaired Ca²⁺ mobilization and sensitization pathways affecting diabetic GI SM dysfunction remain unclear.

The interaction of AGEs/RAGE has been suggested to impair Ca²⁺ mobilization in cardiomyocytes, aortic endothelial cells, and human mesangial cells [15–17]. However, whether GI SM Ca²⁺ mobilization or sensitization is affected by AGEs under diabetic conditions is unknown. The aim of this study was to characterize the effect of AGEs on the key targets within signaling pathways mediating rat colonic SM contraction and MLC phosphorylation *in vivo* and *in vitro*. It has been demonstrated that treatment with AGEs significantly inhibits IP₃R3-operated Ca²⁺ stores release via regulation of the cAMP/PKA-IP₃R3 pathway. In addition, treatment with AGEs significantly decreases acetylcholine (ACh)-induced MYPT1 phosphorylation via regulation of the cAMP/PKA-RhoA/Rho kinase pathway; however, it had no effect on CPI-17 phosphorylation. RAGE is responsible for AGE-induced impairment of Ca²⁺ signaling because blocking RAGE by anti-RAGE-neutralizing antibody reverses the inhibitory effect of AGEs on Ca²⁺ mobilization or sensitization and MLC phosphorylation.

Materials and Methods

Establishment of animal model of diabetes

Forty male Sprague-Dawley rats, weighing 150–180 g, were randomly assigned to four experimental groups, with 10 animals in each group, as follows: control group; control group treated with aminoguanidine

(AG; Sigma, USA); diabetic group; and diabetic group treated with AG. Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ at 60 mg/kg of body weight dissolved in a citrate buffer. Control rats received equal volumes of citrate buffer by i.p. injection. AG (1 g/L) was administered in drinking water from day 1. Diabetes was confirmed 1 week later by the measurement of tail vein blood glucose levels with an Accu-Chek Compact Plus Glucometer (Roche, USA). Only rats with final blood glucose levels >16.7 mmol/L were included in this study. At the end of week 16 following the administration of STZ, the animals were sacrificed after performing distal colonic transit test. The animal care, use, and experimental protocols were approved by the Institutional Animal and Use Committee of Nanjing Medical University (2016-SRFA-064).

Distal colonic transit time

Colonic transit time was measured by using a bead expulsion test [18]. Allyl isothiocyanate (AITC, 0.5% in olive oil, 100 μ L) or vehicle was administered intracolonicly through a catheter. After 5 minutes, a glass bead (5 mm in diameter) was inserted into the colon (3 cm) under transient ether anesthesia and the time until bead expulsion was measured.

Muscle strip contractility assays

The segments of distal colon (whole tissue) from control and diabetic rats were placed in modified Krebs' buffer (37°C, 5% CO₂/95% O₂) under 1-g tension to record isotonic contractions of circular muscle [18]. Tissues were equilibrated (30 minutes), and viability was tested at the beginning and end of experiments by the application of acetylcholine (ACh, 10 μ mol/L, Sigma, USA). Contraction tension was quantified and expressed relative to pretreatment values (normalized to 1).

Assessment of serum AGEs

CML (a biomarker of AGEs) in the serum of control and diabetic rats was determined by enzyme-linked immunosorbent assay (CircuLex, Japan) according to the manufacturer's protocol.

Histological and immunohistochemical analyses

The segments of distal colon from control and diabetic rats were fixed in 4% paraformaldehyde solution for 24 hours and processed for paraffin embedding. The paraffin-embedded samples were sectioned into 5- μ m slices and visualized with hematoxylin/eosin (HE) and immunohistochemical (IHC) staining. The former was used for the measurement of intestinal SM layer thickness and the latter was done by using CML antibodies (1:50; Abcam, USA). The average integrated optical density of CML staining was calculated with Image-Pro Plus software (version 5.0, USA). Five fields were analyzed on each slide.

Preparation and identification of AGE-BSA

A total of 5-g/L BSA (R&D Systems, USA) and 50-mmol/L D-glucose were dissolved in phosphate buffer saline (PBS), dialyzed extensively by ultrafiltration, and then incubated at 37°C for 90 days. The solution was then dialyzed against PBS. BSA solutions without D-glucose were prepared in parallel as controls. No endotoxin was detected in these procedures. The degree of glycation of AGE-BSA was measured using spectrofluorometer at 370 nm excitation and 440 nm emission wavelengths [19].

Cell culture and treatment

SMCs were isolated from colonic tissues and cultured as described earlier [20]. Briefly, whole rat colons were dissected, and the mucosa and serosa were quickly removed from the muscle tissues. After digestion with type II collagenase, SMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) with 10% fetal bovine serum (Gibco). Immunofluorescence staining showed that more than 95% of the cultured cells were positive for SM-specific α -actin (Epitomics, USA) [21, 22]. Cells of passage 2 or 3 were used for the experiments. Prior to the experiments, cells were serum-starved for 24 hours and then treated with 0–200 μ g/mL AGE-BSA for various time intervals in the presence or absence of anti-rat RAGE extracellular domain polyclonal neutralizing antibody (anti-RAGE, 5 μ g/mL; Sigma, USA) and pharmacological inhibitors for PKA (H-89, 15 μ mol/L; Sigma).

Measurement of intracellular Ca²⁺ concentration

The changes in [Ca²⁺]_i in SMCs were monitored by using Fluo-3/AM (Invitrogen, USA), which was initially dissolved in dimethyl sulfoxide (DMSO) and stored at –20°C. The cultured SMCs grown on glass

bottom dishes were rinsed twice with PBS and then incubated in Medium 199 containing 5- μ mol/L Fluo-3/AM in the 95% O₂-5% CO₂ incubator for 40 minutes. After rinsing two more times, the dishes were scanned every 2 seconds with a confocal laser scanning microscope (LSM710, Zeiss, Germany). Fluorescence was excited at a wavelength of 488 nm and emitted light was observed at 515 nm. The variations of [Ca²⁺]_i fluorescence emission intensity were expressed as F/F₀, where F₀ is the intensity of the first imaging.

Western blotting

Distal colonic tissues were dissected from the mucosa and serosa. The remaining tissue containing mainly muscle layers was used for western blotting [23]. The tissues and cultured cells were lysed and centrifuged. Protein samples were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were hybridized with a series of antibodies. Antibodies used for western blotting were SM MHC (1:1000, Abcam), SM α -actin (1:200, Abcam), Cav1.2 (1:500, Millipore, USA), IP₃R3 (1:1000, Cell Signaling, USA), phosphorylated (Ser¹⁷⁵⁵) IP₃R3 (1:1000, Cell Signaling), MLCK (1:200, Santa Cruz, USA), CPI-17 (1:1000, Cell Signaling), phosphorylated (Thr³⁸) CPI-17 (1:1000, Cell Signaling), MYPT1 (1:1000, Cell Signaling), phosphorylated (Thr⁶⁹⁶) MYPT1 (1:1000, Cell Signaling), MLC (1:1000, Cell Signaling), phosphorylated (Ser¹⁹) MLC (1:1000, Cell Signaling), cAMP (1:1000, Abcam), and RAGE (1:500, Abcam). GAPDH (1:5000, Sigma) was used as the internal control.

cAMP/PKA assays

cAMP production in rat colonic muscle layers and SMCs lysate was measured with the cAMP direct immunoassay kit (BioVision, USA) according to the manufacturer's instructions; the PKA activity was assessed by using the PepTag Non-Radioactive Protein Kinase Assay System (Promega, USA) according to the manufacturer's instructions.

RhoA/Rho kinase activity assays

RhoA activity in rat colonic muscle layers and SMCs lysate was assessed by using the G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton, USA) according to the manufacturer's instructions (i.e., by measuring absorbance at 490 nm after indirect immunodetection) [24]. Rho kinase activity was measured by using the Rho Kinase Activity Assay Kit (Cell Biolabs, USA) according to the manufacturer's instructions.

Statistical analysis

All results expressed as mean $\pm$ standard deviation (SD). The differences between the treatment groups were analyzed by using the t-test and SPSS v13.0 (SPSS Inc., USA). A P value <0.05 was considered statistically significant.

Results

Aminoguanidine treatment inhibits the accumulation of AGEs in the serum and colonic muscle tissues of diabetic rats

STZ treatment resulted in a three- to four-fold increase in the blood glucose concentration at 1, 4, 8, and 16 weeks, irrespective of AG treatment. AG-treated control animals had similar blood glucose levels as untreated control animals (Fig. 1A).

Serum levels of CML (AGEs biomarker) were measured to determine the efficacy of AG. The treatment with AG had no effect on serum CML levels in control rats; however, in those with diabetes, a significant increase in serum CML levels were observed at the end of 16 weeks after treatment with STZ compared to that in control rats ($P < 0.01$). However, treatment with AG led to a reduction of serum CML levels to near normal (Fig. 1B). In addition, the administration of AG also prevented CML accumulation and reduced the CML expression in colonic muscle tissues of diabetic rats (Fig. 1C).

Treatment with AG reverses diabetic colon myopathy

The body weight of diabetic rats was significantly reduced compared to that of control groups ($P < 0.01$; Fig. 2A). In contrast, the length of the colon was significantly increased in

Fig. 1. Aminoguanidine treatment inhibits the accumulation of AGEs in the serum and colonic muscle tissues of diabetic rats. **A.** Blood glucose level following an injection of STZ in control and treatment groups. ****** $P < 0.01$ vs. control group, **##** $P < 0.01$ vs. AG group, $n = 10$. **B.** Serum CML levels in the four groups of rats at 16 weeks $*P < 0.05$, ****** $P < 0.01$, $n = 10$. **C.** Representative immunohistochemical staining and western blot images and their quantification of CML accumulation in the colon muscle layers from different groups of rats. Low magnification, $200\times$, scale bar = $100\ \mu\text{m}$; high magnification, $400\times$, scale bar = $50\ \mu\text{m}$; $*P < 0.05$, $n = 10$. AGEs, advanced glycation end products; STZ, streptozotocin; AG, aminoguanidine; CML, N ϵ -carboxymethyl-lysine; IOD, integrated optical density; DM, diabetes mellitus.

diabetic rats, and treatment with AG prevented this phenomenon (Fig. 2B). Morphometric analysis demonstrated that there was a thickened muscle layer in the diabetic colon, whereas AG inhibited this histopathological change (Fig. 2C). Both circular and longitudinal muscle layers were thickened in the diabetic colon (Table 1); however, the ratio between the thicknesses of the two layers did not alter, indicating that the muscle thickened uniformly. To investigate whether the number or volume of SMCs increased, the average number of muscle cells was counted in a unit area. The number of SMCs decreased in the colon of diabetic rats (Table 1), suggesting muscle cells were larger in size than that in controls. However, these changes were partly counteracted by treatment with AG (Table 1).

SM has specialist genes such as SM myosin heavy chain (MHC), SM α -actin, calponin, and caldesmon, among others, which are regulated by similar transcription mechanisms and encode proteins that form the SM contractile apparatus [25, 26]. The expressions of SM MHC and SM α -actin were increased in the colonic muscle tissue of diabetic rats (Fig. 2D); and treatment with AG reversed the protein expression in diabetic rats to normal levels.

Fig. 2. Aminoguanidine treatment reverses diabetic colon myopathy. A. Body weight at 16 weeks following an injection of STZ in control and diabetic groups. ** $P < 0.01$, $n = 10$. B. Length of the colon at 16 weeks. * $P < 0.05$, ** $P < 0.01$, $n = 10$. C. Representative micrographs of HE staining of the colon. Magnification 200 $\times$, scale bar = 100 μm . D. Western blot analysis of SM MHC and α -actin expression in the colon muscle layers. Relative expression was normalized to GAPDH. Upper panel: representative image; lower panel: quantitative analysis from three independent experiments. * $P < 0.05$, ** $P < 0.01$, $n = 10$. STZ, streptozotocin; HE, hematoxylin/eosin; SM, smooth muscle; MHC, myosin heavy chain; AG, aminoguanidine; DM, diabetes mellitus.

Treatment with AG reverses diabetic colon dysmotility

The distal colonic transit time was significantly increased in diabetic rats ($P < 0.05$; Fig. 3A). Colonic circular SM strips from diabetic rats showed weak contractility in response to ACh. The maximum contractile

tension of colonic SM strips in diabetic rats was significantly reduced compared with controls ($P < 0.05$; Fig. 3B), whereas treatment with AG prevented colon motility dysfunction. There was no difference in colonic transit time and contractility of SM strips between the AG-administrated diabetic group and the control group (Fig. 3A and 3B).

Table 1. Histopathological analysis of the colonic muscle layers from control and experimental animals. Data are mean $\pm$ SD. * $P < 0.05$ vs. control group, ** $P < 0.01$ vs. control group, $\Delta P < 0.05$ vs. DM group, $\Delta\Delta P < 0.01$ vs. DM group, $n = 10$. AG, aminoguanidine; DM, diabetes mellitus, SMCs, smooth muscle cells

	Control	Control + AG	DM	DM + AG
Circular (μm)	201.4 $\pm$ 46.0	205.9 $\pm$ 56.2	293.9 $\pm$ 74.6 **	228.1 $\pm$ 36.8 $\Delta\Delta$
Longitudinal (μm)	47.4 $\pm$ 14.2	42.6 $\pm$ 15.4	71.8 $\pm$ 33.6 *	55.5 $\pm$ 9.8
Circular/Longitudinal	4.3 $\pm$ 1.1	4.4 $\pm$ 0.7	4.7 $\pm$ 1.7	4.3 $\pm$ 1.2
Number of SMCs ($\times 10^{-3}$ cells/ μm^2)	6.6 $\pm$ 2.4	6.8 $\pm$ 2.8	3.2 $\pm$ 0.6 **	4.8 $\pm$ 1.3 $\Delta\Delta$

Fig. 3. Aminoguanidine treatment reverses diabetic colon dysmotility. **A.** Distal colonic transit time at 16 weeks following an injection of STZ in diabetic and control groups, $*P < 0.05$, $n = 10$. **B.** Circular muscle strip contractility after exposure to 10^{-3} and 10^{-4} mol/L ACh. Left panel: representative image; right panel: quantification of maximum contractile force of circular muscle strip stimulated by 10^{-3} mol/L ACh. $*P < 0.05$, $n = 10$. **C.** Western blot analysis of phosphorylation of MLC in the colon muscle layers. Relative expression was normalized to GAPDH. Left panel: representative image; right panel: quantitative analysis from three independent experiments. $**P < 0.01$, $n = 10$. STZ, streptozotocin; ACh, acetylcholine; MLC, myosin light chain; AG, aminoguanidine; DM, diabetes mellitus.

Phosphorylation of MLC is a prerequisite for both initial and sustained contraction in SM. Western blot analysis demonstrated that MLC phosphorylation was significantly decreased in colonic muscle tissues of diabetic rats compared to control rats ($P < 0.01$), but was not decreased in AG-treated diabetic rats (Fig. 3C).

Diabetic rats showed significant changes in levels of Ca^{2+} signaling proteins that can be reversed by AG

The possibility that impaired Ca^{2+} mobilization and sensitization are responsible for the reduced contractility of colon SM in diabetic rats was investigated. Western blot analysis revealed that L-type Ca^{2+} channel (Cav1.2), IP_3R3 , and MLCK protein expression in the colonic muscle tissues were not affected by diabetes (Fig. 4A). Because IP_3R3 phosphorylation is involved in the inhibition of IP_3R3 -mediated Ca^{2+} release [27–29], it was examined whether IP_3R3 phosphorylation is increased in the colonic muscle tissues of diabetic rats. We found that phosphorylation of IP_3R3 was significantly increased in diabetic rats ($P < 0.01$), whereas treatment with AG inhibited this increase (Fig. 4A). We then examined whether the activity of Ca^{2+} sensitization and contractile proteins are altered in the colonic muscle tissues of diabetic rats. As shown in Fig. 4B, RhoA and Rho kinase activities decreased by 20–40%

Fig. 4. Aminoguanidine treatment restored the levels of Ca²⁺ signaling proteins in diabetic rats. **A.** Western blot analysis of Ca²⁺ mobilization proteins in the colon muscle layers from control and treated rats. Relative expression was normalized to GAPDH. Left panel: representative image; right panel: quantitative analysis from three independent experiments. **P<0.01, n = 10. **B.** Activity and expression of Ca²⁺ sensitization proteins in the colon muscle layers from different groups. Left panel: RhoA and Rho kinase activity; right panel: representative western blot image of phosphorylation of MYPT1 and CPI17; quantitative analysis from three independent experiments. *P<0.05, **P<0.01, n = 10. **C.** Content of cAMP and activity of PKA from different groups. **P<0.01, n = 10. MYPT1, myosin phosphatase target subunit protein-1; IP₃R3, inositol 1,4,5-trisphosphate receptor type 3; MLCK, myosin light chain kinase; AG, aminoguanidine; DM, diabetes mellitus.

in diabetic rats. Phosphorylation of MYPT1 was also significantly inhibited in diabetic rats; however, CPI-17 phosphorylation was not affected. The administration of AG prevented these changes in diabetic rats (Fig. 4B).

It has been reported that cAMP/PKA phosphorylates IP₃R3 in SMCs and inhibits IP₃R3-operated Ca²⁺ store release [30]. In addition, cAMP/PKA decreases MLC phosphorylation by inhibiting RhoA, thus activating MLCP [31, 32]. It was examined whether diabetes or AG affects the expression and activity of cAMP/PKA. We found that cAMP production and PKA activity were significantly increased in the colonic muscle tissues of diabetic rats compared to controls (P < 0.01; Fig. 4C), and treatment with AG effectively attenuated these changes (Fig. 4C).

AGE-BSA inhibits MLC phosphorylation and Ca²⁺ release from IP₃R3-operated Ca²⁺ stores in isolated colonic SMCs

Colonic SMCs were successfully isolated from normal rat colon and identified by immunofluorescence staining with antibody to α -actin (data not shown). Phosphorylation of MLC is a prerequisite for both initial and sustained contractions in SM and is regulated by the balance between MLCK and MLCP activities. Western blot analysis demonstrated that

Fig. 5. AGE-BSA inhibits IP₃R3-operated Ca²⁺ stores release and MLC phosphorylation in isolated rat colonic SMCs. **A.** SMCs were treated with 200-µg/mL BSA and different doses (50, 100, 150, and 200 µg/mL) of AGE-BSA for 1 hour. Western blot analysis of phosphorylation of MLC in SMCs stimulated by 0.1 µM Ach for 30 seconds. **B.** Changes in fluorescence intensity due to [Ca²⁺]_i relative to baseline (F/F₀) in SMCs treated with 200-µg/mL BSA and 150-µg/mL AGE-BSA. F₀ was derived from the averaged intensity of the first 0–30 seconds. SMCs were incubated in normal Krebs (Ca²⁺-replete) solution. Left panel: fluorescence intensity of [Ca²⁺]_i; right panel: quantitative analysis of peak F/F₀ and representative image of fluorescence at peak F/F₀ from three independent experiments. ***P<0.001, n = 3. **C.** Changes in fluorescence intensity due to [Ca²⁺]_i relative to baseline (F/F₀) and phosphorylation of IP₃R3 in SMCs treated with 200-µg/mL BSA and 150-µg/mL AGE-BSA for 1 hour. SMCs were incubated in Ca²⁺-free buffer solutions. Left panel: fluorescence intensity of [Ca²⁺]_i and quantitative analysis of peak F/F₀ from three independent experiments; right panel: representative western blot image of phosphorylation of IP₃R3. **P<0.01, ***P<0.001, n = 3. **D.** Changes in fluorescence intensity due to [Ca²⁺]_i relative to baseline (F/F₀) in SMCs treated with 200-µg/mL BSA and 150-µg/mL AGE-BSA. SMCs were incubated in normal Krebs (Ca²⁺-replete) solution with thapsigargin. Upper panel: fluorescence intensity of [Ca²⁺]_i; lower panel: quantitative analysis of peak F/F₀ from three independent experiments. n = 3. AGE-BSA, advanced glycation end product-bovine serum albumin; Ach, acetylcholine; IP₃R3, inositol 1,4,5-trisphosphate receptor type 3; MLC, myosin light chain, SMCs, smooth muscle cells; F, fluorescence.

treatment with AGE-BSA for 1 hour caused a significant decrease in MLC phosphorylation in SMCs stimulated with 0.1 µM Ach for 30 seconds; non-glycated BSA (200 µg/mL) had no effect on the phosphorylation of MLC (Fig. 5A). AGE-BSA at 150 µg/mL was considered the most effective concentration and was used in follow-up experiments.

The effect of AGE-BSA on Ca²⁺ mobilization *in vitro* was then investigated. In SMCs incubated with a control and BSA sample, the subsequent exposure to Ach caused a rapid increase in intracellular [Ca²⁺]_i consisting of a transient increase in fluorescence ratio (peaking at approximately two-fold the normalized basal fluorescence ratio), followed by a sustained increase at about 1.5 times the basal level (Fig. 5B). The exposure of SMCs to AGE-

BSA significantly attenuated the ACh-induced increase in $[Ca^{2+}]_i$ compared with the control and BSA groups, and the intracellular $[Ca^{2+}]_i$ gradually returned to the basal level (Fig. 5B).

The effect of AGE-BSA on the different components of Ca^{2+} mobilization was investigated further by superfusing the SMCs with Ca^{2+} -free buffer solutions. In the absence of extracellular Ca^{2+} , all increase in intracellular $[Ca^{2+}]_i$ was owing to the release of Ca^{2+} from intracellular stores only. Under these conditions, AGE-BSA inhibited ACh-induced release of intracellular Ca^{2+} (Fig. 5C). Because IP_3R3 phosphorylation inhibits only IP_3R3 -mediated intracellular Ca^{2+} release [27–29], the effect of AGE-BSA on IP_3R3 phosphorylation was further investigated. AGE-BSA stimulation resulted in significantly increased phosphorylation of IP_3R3 ($P < 0.001$; Fig. 5C).

Additional experiments were subsequently performed to examine the effects of AGE-BSA on extracellular Ca^{2+} influx. SMCs were incubated in normal Krebs (Ca^{2+} -replete) solution with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase pump that causes the emptying of intracellular Ca^{2+} stores, and ionomycin, which acts directly on the intracellular Ca^{2+} storage organelle to empty the store. In the absence of intracellular Ca^{2+} , AGE-BSA did not affect Ca^{2+} entry; i.e., the transient increase in peak fluorescence ratio was not significantly different from the BSA group or the control group (Fig. 5D). Collectively, these data supported a specific effect of AGE-BSA on Ca^{2+} release from the IP_3R3 -operated Ca^{2+} stores in the endoplasmic reticulum, without affecting extracellular Ca^{2+} entry.

AGE-BSA downregulates RhoA/Rho kinase activity and MYPT1 phosphorylation

The sustained phase of SM contraction is Ca^{2+} -independent and involves RhoA activation, resulting in the inhibition of MLCP via Rho kinase-mediated phosphorylation of MYPT1 and PKC-mediated phosphorylation of CPI-17 [12]. To identify the signaling target that mediates the AGE-BSA-induced inhibitory effect on sustained contraction, the activity of RhoA/Rho kinase and the phosphorylation of MYPT1 were examined. As shown in Fig. 6A and 6B, AGE-BSA exposure caused a significant decrease in RhoA and Rho kinase activities ($P < 0.05$), and reduced the phosphorylation of MYPT1 ($P < 0.01$) compared with control. This likely enhanced the MLCP activity, dephosphorylates MLC, and causes relaxation of SMCs.

The expression and phosphorylation of CPI-17 were then examined by western blot analysis. As shown in Fig. 6B, there were no differences in the expression or phosphorylation of

Fig. 6. AGE-BSA down-regulates RhoA/Rho kinase activity and MYPT1 phosphorylation in SMCs. A. Activity of RhoA/Rho kinase in SMCs treated with 200- μ g/mL BSA and 150- μ g/mL AGE-BSA for 1 hour, * $P < 0.05$, ** $P < 0.01$, $n = 3$. B. Western blot analysis of phosphorylation of MYPT1 and CPI17 in SMCs treated with 200- μ g/mL BSA and 150- μ g/mL AGE-BSA. Left panel: representative image; right panel: quantitative analysis from three independent experiments. ** $P < 0.01$, $n = 3$. AGE-BSA, advanced glycation end product-bovine serum albumin; MYPT1, myosin phosphatase target subunit protein-1; SMCs, smooth muscle cells.

CPI-17 in SMCs between the control and AGE-BSA-treated groups. These data demonstrated that the inhibitory effect of AGE-BSA on sustained contraction might be due to targeting of the RhoA/Rho kinase/MYPT1 pathway, but not CPI-17.

cAMP/PKA mediates AGE-BSA-induced impairment of Ca^{2+} mobilization and sensitization and MLC phosphorylation

Because cAMP/PKA is an essential negative regulator of both IP_3R3 and RhoA [30–32], it was assumed that AGEs impair Ca^{2+} signaling in SMCs by activating cAMP/PKA signaling. As shown in Fig. 7A, AGE-BSA at every concentration significantly increased cAMP production compared with the BSA and control groups. The activity of PKA, a direct downstream target protein of cAMP, was then tested. AGE-BSA stimulation caused a significant increase in the PKA activity. The stimulation effect was displayed in a concentration-dependent fashion, which closely paralleled the production of cAMP (Fig. 7A).

The effect of PKA on AGE-BSA-induced impairment of Ca^{2+} signaling and MLC phosphorylation was then tested. The PKA inhibitor H-89 attenuated the AGE-BSA-induced

Fig. 7. cAMP/PKA mediates AGE-BSA-induced impairment of Ca^{2+} mobilization and sensitization and MLC phosphorylation. A. Content of cAMP and activity of PKA in SMCs treated with 200-µg/mL BSA and different concentrations of AGE-BSA. ** $P < 0.01$, *** $P < 0.001$, $n = 3$. B. Representative western blot image of phosphorylation of IP_3R3 , MYPT1 and MLC in SMCs treated with 15-µmol/L H-89 for 1 hour, or untreated, followed by treatment with 200-µg/mL BSA and 150-µg/mL AGE-BSA for 1 hour. C. Activity of RhoA/Rho kinase in SMCs treated with 15-µmol/L H-89 for 1 hour, or untreated, followed by treatment with 200-µg/mL BSA and 150-µg/mL AGE-BSA for 1 hour. * $P < 0.05$, ** $P < 0.01$, $n = 3$. AGE-BSA, advanced glycation end product-bovine serum albumin; MLC, myosin light chain; SMCs, smooth muscle cells; IP_3R3 , inositol 1,4,5-trisphosphate receptor type 3; MYPT1, myosin phosphatase target subunit protein-1.

MLC dephosphorylation to a level that was almost similar to the BSA and control groups (Fig. 7B). Moreover, the inhibition of PKA attenuated the AGE-BSA-induced phosphorylation of IP₃R3 (Fig. 7B), and restored the RhoA/Rho kinase activities and MYPT1 phosphorylation to control levels (Fig. 7B, 7C). These findings strongly suggested that AGEs impair Ca²⁺ signaling via activation of the cAMP/PKA pathway.

RAGE mediates AGE-BSA-induced activation of the cAMP/PKA pathway and impairment of Ca²⁺ mobilization and sensitization

First, double immunofluorescence staining was used to detect RAGE protein expression in colonic SMCs. As shown in Fig. 8A, the nuclei in the cultured cells were identified by Hoechst staining (blue), cells also exhibited α -actin immunoreactivity (red); immunostaining

Fig. 8. RAGE mediates AGE-BSA-induced activation of the cAMP/PKA pathway and impairment of Ca²⁺ mobilization and sensitization. A. Representative double immunofluorescence staining of SMCs for α -actin and RAGE. Nuclei were identified by Hoechst staining. Scale bar = 20 μ m. B. Content of cAMP and activity of PKA in SMCs treated with 0.5-mg/mL RAGE-neutralizing antibody for 24 hours, or untreated, followed by treatment with 200- μ g/mL BSA and 150- μ g/mL AGE-BSA for 1 hour. **P<0.01, ***P<0.001, n = 3. C. Representative western blot image of phosphorylation of MLC in SMCs treated with 0.5-mg/mL RAGE-neutralizing antibody for 24 hours, or untreated, followed by treatment with 200- μ g/mL BSA and 150- μ g/mL AGE-BSA for 1 hour. RAGE, receptor for advanced glycosylated end products; AGE-BSA, advanced glycation end product-bovine serum albumin; SMCs, smooth muscle cells; MLC, myosin light chain.

for RAGE was evident. These data confirmed the presence of RAGE in colonic SMCs.

The role of RAGE in AGEs-related effects was then tested. The RAGE-neutralizing antibody significantly decreased the AGE-BSA-mediated increase in cAMP production and PKA activation (Fig. 8B). RAGE inhibition also reversed the AGE-BSA-mediated reduction in MLC phosphorylation (Fig. 8C). Moreover, the inhibition of RAGE attenuated the AGE-BSA-induced phosphorylation of IP₃R3 (Fig. 8C), and restored the RhoA/Rho kinase activities and MYPT1 phosphorylation to control levels (Fig. 8C, 8D). Together, these findings strongly suggested that AGEs impair Ca²⁺ signaling in colon SMCs through the activation of the cAMP/PKA pathway via interaction with RAGE.

Discussion

The possible effect of AGEs on intestinal SMCs Ca²⁺ signaling that results in colonic dysmotility in DM has been presented in this study. It was found that the inhibition of AGE formation ameliorates colonic dysmotility in diabetic rats. *In vitro*, AGEs inhibited Ca²⁺ mobilization from the endoplasmic reticulum and decreased Ca²⁺ sensitization, thereby decreasing MLC phosphorylation and contraction of SMCs. It was further demonstrated that the inhibition of cAMP/PKA signaling reverses AGE-induced Ca²⁺ mobilization and sensitization impairment, indicating that the cAMP/PKA pathway plays a role in intestinal SM dysfunction in DM.

AGEs, the complex and heterogeneous group of compounds formed by non-enzymatic reaction between the reducing sugar and amino groups of proteins, have been implicated in diabetes-related complications [33]. Increasing evidence suggests that AGEs, along with their receptor (RAGE), are involved in diabetic GI dysmotility [10, 11]. In this study, it was found that the serum levels of AGEs were significantly increased along with increased AGEs expression in the distal colon muscle layers in the rat model of DM. The findings provide further evidence of a link between AGEs and colonic dysmotility in DM.

The intestinal contraction process is mainly carried out by SMCs [12]. However, a few studies have explored the possible effects of diabetes directly on colonic SM. It was observed that diabetic rats showed a longer length of the colon, thickened muscle wall, reduced muscle cell number, and increased expression of contractile function proteins MHC and SM α -actin, whereas the expression or activities of Ca²⁺ signaling proteins decreased. These results suggest that diabetic colon dysmotility may be owing to myopathy, and is more related to a Ca²⁺ signaling disorder than cell phenotype modulation of SMCs similar to that seen in a previous study [7].

AG, an inhibitor of AGE-mediated crosslinking, has been shown to prevent diabetic complications in animal models [34, 35]. In the experiments carried out in this study, AG successfully reduced the accumulation of AGEs in the serum and colonic muscle tissues of STZ-induced diabetic rats, without affecting blood glucose levels. The administration of AG also reversed diabetic colonic myopathy. Therefore, it may be hypothesized that AGEs could be the cause of diabetic colonic dysmotility.

AGEs have been suggested to impair Ca²⁺ mobilization in several cell types [15–17]. Mene et al. reported that AGEs reduced cytosolic [Ca²⁺]_i in human mesangial cells and the supplementation of AG prevented the downregulation of [Ca²⁺]_i signaling [17]. In addition, the effects of AGEs on [Ca²⁺]_i are not restricted to the release of Ca²⁺ from the intracellular stores, as AGEs can also reduce Ca²⁺ influx through plasma membrane channels. In this study, cultured rat colon SMCs were exposed *in vitro* to AGE-BSA for 60 minutes prior to the measurement of cytosolic [Ca²⁺]_i. AGE-BSA-inhibited IP₃R3-mediated mobilization of intracellular Ca²⁺ in SMCs incubated with Ca²⁺-free buffer solutions. However, AGE-BSA failed to influence extracellular Ca²⁺ entry in SMCs incubated with Ca²⁺-containing buffer solutions and drugs (thapsigargin and ionomycin) that cause the emptying of intracellular Ca²⁺ stores. The inhibition of intracellular Ca²⁺ mobilization may reduce the activity of MLCK and the phosphorylation of MLC, thereby reducing SM contractility.

The sustained phase of SM contraction is Ca^{2+} -independent and involves RhoA activation, which then inhibits MLCP via Rho kinase-mediated phosphorylation of MYPT1, the regulatory subunit of MLCP [12]. Our data showed that AGE-BSA treatment decreased RhoA/Rho kinase activities and MYPT1 phosphorylation. In turn, this likely enhances the MLCP activity and dephosphorylates MLC, resulting in the relaxation of SMCs.

Furthermore, we sought to gain a deeper insight into the molecular mechanisms by which AGEs regulate Ca^{2+} mobilization and sensitization in colon SMCs. Previous studies have demonstrated that the cAMP/PKA pathway is activated by AGEs in diabetes [36]. Moreover, the cAMP/PKA pathway is widely recognized to regulate aortic SM contraction [37, 38]. In gastric SMCs and intact aorta, PKA-mediated phosphorylation of $\text{IP}_3\text{R3}$ decreases the number of binding sites for IP_3 on $\text{IP}_3\text{R3}$ and thus attenuates $\text{IP}_3\text{R3}$ -mediated Ca^{2+} release [29]. There is also some evidence that ion channels are involved in PKA-mediated SMCs relaxation [38, 39]. Other studies have demonstrated that the cAMP/PKA pathway may inactivate RhoA through phosphorylation, thereby stopping its inhibitory effect on MLCP, and allowing MLCP to dephosphorylate MLC and relax vascular SM independent of the intracellular Ca^{2+} level [40]. The data from this study showed that both cAMP and PKA expressions could be upregulated in SMCs by AGE-BSA treatment. Then we used H-89 to inhibit the activity of PKA. H-89 is the most effective inhibitor of PKA and it inhibits PKA with an inhibition constant of 0.05 μM . At a concentration of 10 μM it inhibits PKA by 80–100% [41]. It acts as competitive antagonist of ATP at the ATP-binding site on the PKA catalytic subunit. ATP is required to phosphorylate appropriate serine or threonine residues on target proteins; therefore, blockade of this site prevents the cAMP dependent phosphorylation of PKA substrates [42]. H-89 also inhibits several other kinases like S6K1, MSK1, PKB α and MAPKAP-K1bm [43]. The inhibition of PKA attenuated the effect of AGE-BSA-induced phosphorylation of $\text{IP}_3\text{R3}$. In parallel, the inhibitory effect of AGE-BSA on RhoA/Rho kinase activities and MYPT1 phosphorylation were reversed, verifying that the cAMP/PKA pathway contributes to AGEs-induced Ca^{2+} mobilization and sensitization impairment in diabetes.

An accelerated expression of RAGE mediated by AGE may contribute to oxidative stress, which plays a key role in the pathogenesis of diabetic complications [44, 45]. In human cardiomyocytes, blocking RAGE with anti-RAGE IgG completely abolished the AGE-induced depletion of sarcoplasmic reticulum Ca^{2+} content, which may contribute to contractile dysfunction in diabetic cardiomyopathy [46]. In this study, RAGE inhibition reversed AGE-BSA-mediated $\text{IP}_3\text{R3}$ phosphorylation and AGE-BSA-mediated reduction in RhoA/Rho

Fig. 9. Proposed mechanism by which AGEs interfere with Ca^{2+} signaling in colonic smooth muscle cells. AGEs activate RAGE, which results in an increased activation of cAMP/PKA, an essential negative regulator of both $\text{IP}_3\text{R3}$ and RhoA. This leads to the inhibition of $\text{IP}_3\text{R3}$ -mediated Ca^{2+} release and initial contraction via the cAMP/PKA- $\text{IP}_3\text{R3}$ pathway. In addition, AGEs can also decrease Ca^{2+} sensitization and sustained contraction via the cAMP/PKA-RhoA/Rho kinase pathway. AGEs, advanced glycation end products; RAGE, receptor for advanced glycated end products; $\text{IP}_3\text{R3}$, inositol 1,4,5-trisphosphate receptor type 3; MLCK, myosin light chain kinase; MLC, myosin light chain; MYPT1, myosin phosphatase target subunit protein-1; MLCP, myosin light chain phosphatase.

kinase activities and MYPT1 phosphorylation. Together, these findings strongly suggest that AGEs impair Ca^{2+} signaling in colon SMCs by interacting with RAGE.

There are some limitations to the investigation carried out in this study. First, the possible effects of AGEs on Ca^{2+} release from the endoplasmic reticulum via other receptors, such as the ryanodine receptor, need to be further investigated. Second, the intracellular $[\text{Ca}^{2+}]_i$ was measured only by laser scanning confocal microscopy. This technique can be combined with other experimental methods such as flow cytometry and patch clamp technique in future studies.

In conclusion, AGEs impair Ca^{2+} signaling in colon SMCs through the activation of the cAMP/PKA pathway via interacting with RAGE, which results in a reduction of MLC phosphorylation and contractility (Fig. 9). Whether AGEs inhibit Ca^{2+} mobilization and sensitization via other mechanisms is yet to be determined. However, this study suggests a possible mechanism for GI dysmotility and facilitates further understanding of the role of Ca^{2+} mobilization and sensitization pathways in the pathophysiology of diabetic GI SM dysfunction.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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