

Original Article

Advanced glycation end products inhibit distal colon contraction in rats via protein kinase C-dependent modulation of intracellular calcium

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Abstract: Aims: To investigate the effect of advanced glycation end products (AGEs) on contractile activity of rat distal colon and its possible mechanisms. Methods: Contractile responses of colonic smooth muscle strips from male SD rats were recorded using a polyphysiograph. Colonic smooth muscle cells were isolated and identified by immunofluorescence. Changes in $[Ca^{2+}]_i$ in response to AGEs were measured by confocal laser scanning microscopy. Immunoprecipitation and Western blotting were used to examine the phosphorylation of type 3 $InsP_3$ receptors ($InsP_3R3$) in colonic smooth muscle cells. The PKC inhibitor chelerythrine and the PKC activator phorbol 12-myristate 13-acetate (PMA) was used to examine the role of PKC in the responses to AGEs. Results: Carbachol-induced contractions of colonic smooth muscle strips were relaxed following the administration of 150 μ g/mL and 200 μ g/mL AGEs compared to control, and this was significantly counteracted by prior administration of chelerythrine and decreased more significantly by prior administration of PMA before exposure to AGEs. AGEs at the concentration of 150 μ g/mL or 200 μ g/mL significantly inhibited $[Ca^{2+}]_i$ compared with the control group. AGEs at 150 μ g/mL was considered as the most effective concentration in vitro, but this effect was less marked in the presence of chelerythrine. PMA (1 μ M) significantly enhanced AGEs-mediated effect on the decrease of $[Ca^{2+}]_i$. AGEs increased PKC activity compared to controls, and increased phosphorylation of $InsP_3R3$. The latter effect was prevented by chelerythrine. Conclusions: AGEs inhibit contraction of colonic smooth muscle strips and reduce $[Ca^{2+}]_i$ in colonic smooth muscle cells. The mechanism involves the release of $InsP_3R3$ -operated Ca^{2+} stores, regulated by the PKC signaling pathway.

Keywords: Advanced glycation end products, colon muscle contraction, colonic smooth muscle cell, protein kinase C, calcium modulation

Introduction

In recent years, an increasing number of gastrointestinal (GI) complications such as constipation, dysphagia, reflux, and nausea have been seen in diabetic patients. The prevalence of GI dysfunction was found to be more common in patients with long-term diabetes. Seventy-five percent of patients with diabetes mellitus suffered from these symptoms [1, 2]. The pathogenesis of GI dysfunction in diabetes is usually multifactorial: it may result from autonomic neuropathy, the interstitial cells of Cajal (ICC) and/or smooth muscle cell lesions [3-5]. Smooth muscle contraction is regulated by

phosphorylation of the 20 kDa myosin light chain (MLC20), which is in turn regulated by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). This process can be initiated by increased levels of calcium, which binds to calmodulation proteins, then activates MLCK. Activated MLCK phosphorylates the 20 kDa myosin light chain (MLC20), which promotes the cross-bridge cycle between actin filaments and myosin heads, leading to smooth muscle contraction. Under physiological conditions, excitation-contraction coupling is largely due to the classic mechanism of calcium sensitization of smooth muscle contraction. When MLC20 is dephosphorylated by

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MLCP, cross-bridge cycling is reduced, leading to muscle relaxation [6, 7]. In the present study, AGEs in serum and various tissues from diabetic patients were significantly increased compared to the general population. AGEs are known to be an important risk factor for diabetic complications such as diabetic nephropathy and diabetic angiopathy. However, the correlation of AGEs with GI dysfunction in diabetes mellitus has not been studied [8-10]. In addition, it has been reported that abnormalities of the intracellular calcium signaling pathway lead to abnormal calcium concentrations in colonic smooth muscle cells, as well as other cells, in diabetes mellitus [11]. The purpose of this study was to investigate whether AGEs affected the contractile tension of colonic smooth muscle strips and the possible mechanism. It provides a basis for further study of the mechanisms involved in gastrointestinal dysmotility in diabetes patients.

Materials and methods

Preparation of cells and cell culture

Male SD rats (weighing 200-250 g) were used for the experiments. The animals were killed by cervical dislocation. Smooth muscle cells (SMCs) were isolated by enzymatic digestion from colonic tissues and cultured as described previously. The whole SD rat colon was excised and the contents were washed away with ice-cold HEPES-Ringer. The mucosa and serosa were quickly dissected from muscle tissues, and the tissue was cut into 0.5 cm segments. The segments were transferred into a centrifuge tube and dispersed with an enzyme solution containing 1 mg/mL collagenase type II and 2 mg/ml trypsin inhibitor. The tube was incubated at 37°C for 30 min. An equal volume DMEM containing 10% fetal bovine serum was added to stop digestion. Following completion of digestion, SMCs were cultured in DMEM containing 10% fetal bovine serum. Cells were stored in an incubator at 37°C, 95% O₂, and 5% CO₂. Cells of passage 2 or 3 were used for the studies.

Immunofluorescence to identify SMCs

Cultured SMCs were fixed in ice-cold acetone, and then incubated in 10% goat serum for 1 h to reduce nonspecific antibody binding. Cultured SMC were then incubated overnight at

4°C with antibody to α -actin to identify smooth muscle cells. Alexa Fluor 488-conjugated anti-rabbit IgG was used as secondary antibody and nuclei were stained with Hoechst 33258. Immunoreactivity was examined under a confocal laser scanning microscope (LSM710, Zeiss, Germany) at an excitation wavelength of 488 nm.

Measurement of intracellular Ca²⁺ concentration

Cultured SMCs were placed on glass-bottomed dishes. The dishes were washed twice with PBS and then loaded with Fluo3/AM (5 μ mol/L) for 40 min in the 95% O₂/5% CO₂ incubator. Following two more rinses, the dishes were scanned using a confocal laser scanning microscope. Fluorescence intensity (F) was measured at 488nm using a fluorometer and therefore intracellular Ca²⁺ concentration ([Ca²⁺]_i) following addition of AGEs was expressed as F/F₀, where F₀ was the intensity at baseline.

Detection of protein kinase C (PKC) activity

Samples were added to appropriate wells of the PKC substrate microtiter plate. The reaction was initiated by adding 10 μ l of diluted ATP to each well, followed by incubation with 40 μ l of phosphospecific contents for 60 min and then 40 μ l of diluted anti-rabbit IgG-GRP conjugate for 30 min. Finally, 20 μ l of acid stop solution was added to each well and the absorbance at 450 nm was measured.

Immunoprecipitation and immunoblotting

Cultured cells were lysed on ice for 30 min and centrifuged at 12000 rpm for 20 min. Protein samples for immunoprecipitation were incubated with a 1:100 dilution of an InsP₃R3-specific monoclonal antibody at 4°C for 2 h. Immobilized protein A beads were added to each sample for 1 h at 4°C. Following immunoprecipitation of InsP₃R3, proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes for 1 h at 100 V. The membranes were blocked with 5% skim milk for 1 h at room temperature, and then incubated with primary antibodies (1:1000) at 4°C overnight. Phospho-(Ser/Thr) substrate antibody specifically detected phosphorylated Ser/Thr residues with Arg at the -2 or -3 position within the PKC substrate

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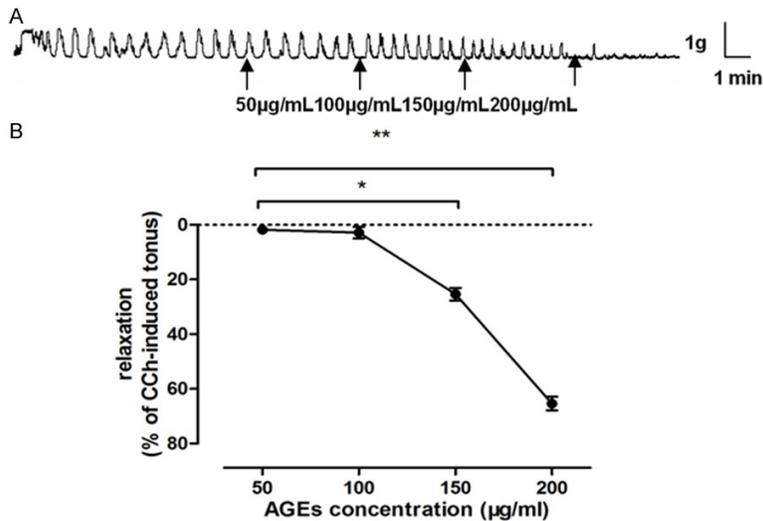


Figure 1. Effects of different concentrations of advanced glycation end products (AGEs) on smooth muscle strips from adult rat colon. Carbachol-induced contractions of colonic smooth muscle strips were relaxed following the administration of 150 µg/mL and 200 µg/mL AGEs compared to control (25.41%±2.32%, 65.42%±2.52% vs. 1.08%±0.41%, * P <0.05, ** P <0.01).

sequence. The membranes were probed with corresponding horseradish peroxidase-conjugated secondary antibody at 1:4000 dilutions for 1 h at 37°C. Protein bands were detected with enhanced chemiluminescence.

Measurement of colonic smooth muscle contraction

Distal colons were dissected (about 5 cm from the anus) and stored on ice for less than 1 h. The mucosal layers were removed by microdissection under a magnifying glass. One end of the strip was fixed to a hook on the bottom of the chamber, while the other end was fixed to an isometric force transducer (Alcott Biotech, Norcross, GA, USA). Each fresh smooth muscle strip was placed in a warm, oxygenated organ bath, which contained 10 mL Tyrode's buffer constantly warmed by circulating water at 37°C and bubbled with carbogen (95% O₂ and 5% CO₂). The strips were allowed to equilibrate for 1 h under an initial tension of 1 g, and the solution was changed every 20 min. Contractile amplitude and frequency in response to carbachol alone (control values) were recorded and compared with the response to treatment (response values). At least three muscle strips from the distal colon of each rat were used for these experiments. The results are presented as the change in percentage (change in per-

centage = 100% × (control value - response value)/control value).

Drugs and solutions

Preparation of AGEs-BSA: The 50 mg/mL bovine serum albumin (BSA) and 0.5 mol/L glucose were put in 0.2 mol/L phosphate buffer (PBS, pH 7.4), 37°C for 3 months, to form AGEs-BSA. At the same time, the BSA (O-BSA) was prepared by parallel conditions without adding glucose. Dialysis bags with pore size of molecular weight 10000 were used to remove the non-reactive glucose, in 0.01 mol/L PBS for 24 h. Then it was sterilized with 0.22 M filter. Protein concentrations

were determined by BCA, then preserved in -20°C.

Other drugs: Type II collagenase, phorbol 12-myristate 13-acetate, chelerythrine, and were obtained from Sigma. DMEM, fetal bovine serum, and penicillin-streptomycin were obtained from Gibco, USA. Antibodies to α -actin and InsP₃R3 were obtained from Cell Signaling Technology. Protein A beads were obtained from Biosciences Transduction Laboratories. Hoechst 33258 from Bioworld and Alexa Fluor 488 and Fluo-3/AM from Invitrogen.

Statistical analysis

Data were expressed as mean ± SD. The differences between two groups were analyzed using the Student's t test or ANOVA. Graphpad 5.0 was used for charting. Differences between two groups with values of P <0.05 were considered statistically significant.

Results

Effects of AGEs on colonic smooth muscle strips

Carbachol-induced contraction of colonic smooth muscle strips were relaxed following the administration of 150 µg/mL or 200 µg/mL AGEs compared to control (25.41%±2.32%,

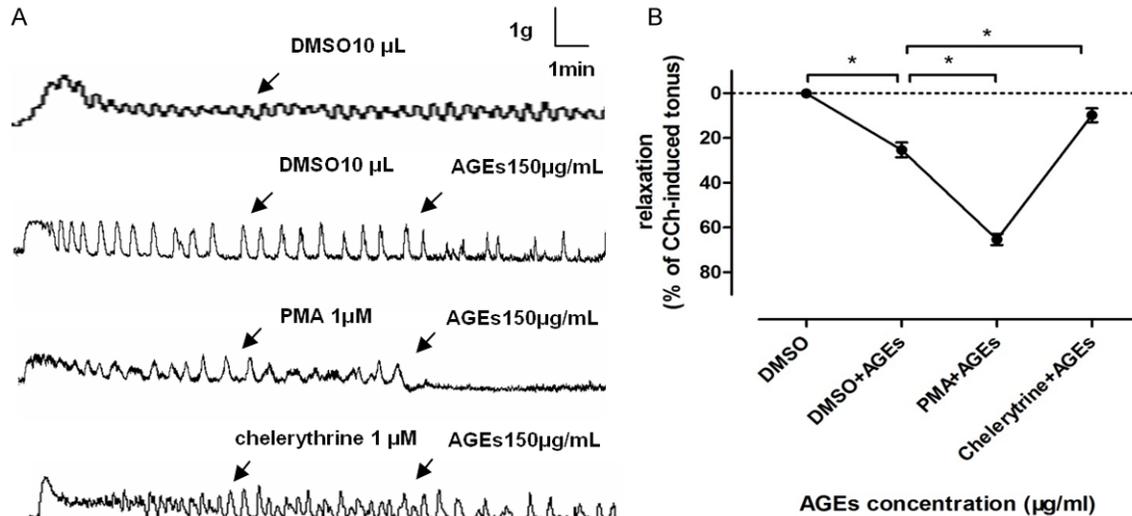


Figure 2. AGEs-induced inhibition of the contractions of colonic smooth muscle strips could be partially prevented by pretreatment with chelerythrine (9.98%±3.12% vs. 25.43%±3.31%, **P*<0.05) and decreased more significantly by prior administration of PMA before exposure to AGEs (65.61%±2.76% vs. 25.43%±3.31%, **P*<0.05).

65.42%±2.52% vs. 1.08%±0.41%, *P*<0.05). AGEs affected the amplitude but not the frequency of the contractions of the strips when applied for 2-3 min (**Figure 1**). The AGEs-induced inhibition of colonic smooth muscle contraction was significantly counteracted by prior administration of chelerythrine (9.98%±3.12% vs. 25.43%±3.31%, *P*<0.05) and decreased more significantly by prior administration of PMA before exposure to AGEs (65.61%±2.76% vs. 25.43%±3.31%, *P*<0.05), (**Figure 2**).

Identification of cultured SMCs

Colonic smooth muscle cells were successfully isolated from normal rat colon and identified by immunofluorescence staining with antibody to α -actin. They were characterized by distinctive fusiform shapes and cytoplasmic red fluorescence (**Figure 3**).

Effects of AGEs on intracellular Ca^{2+} in SMCs

As shown in **Figure 4**, AGEs inhibited $[Ca^{2+}]_i$ in a concentration-dependent manner. AGEs at the concentration of 150 μ g/mL or 200 μ g/mL significantly inhibited $[Ca^{2+}]_i$ compared with the control group (0.37±0.04, 0.29±0.05 vs. 0.01±0.03, *P*<0.05). AGEs at 150 μ g/mL was considered as the most effective concentration in vitro. Pretreatment with chelerythrine (1 μ M) reduced the inhibitory effects of AGEs on $[Ca^{2+}]_i$ (0.13±0.03 vs. 0.37±0.03, *P*<0.05). Pretreat-

ment with PKC activator PMA (1 μ M) significantly enhanced AGEs-mediated effect on the decrease of $[Ca^{2+}]_i$ (0.49±0.03 vs. 0.37±0.03, *P*<0.05). Each experiment was repeated at least three times. The results suggest that the effects of AGEs are due to regulation of the PKC pathway (**Figure 5**).

PKC activity was increased by AGEs

PKC activity increased in SMCs treated with 150 μ g/mL and 200 μ g/mL AGEs compared with the control group (2.36±0.07, 2.10±0.06 vs. 0.51±0.06, *P*<0.01; **Figure 6**).

AGE stimulation resulted in PKC-dependent phosphorylation of $InsP_3R3$

There results of immunoprecipitation studies using cultured SMCs are shown in **Figure 7**. AGEs stimulation resulted in increased phosphorylation of $InsP_3R3$, which was prevented by the PKC inhibitor chelerythrine (0.34±0.02 vs. 1.35±0.10, *P*<0.05). The PKC activator phorbol 12-myristate 13-acetate (PMA; 1 μ mol/L), used as a positive control, also resulted in enhanced phosphorylation of $InsP_3R3$ (1.39±0.10 vs. 0.98±0.02, *P*<0.05). All samples were treated for 5 min (**Figure 7**).

Discussion

The results of this study demonstrate that (1) AGEs inhibited the contraction of colonic

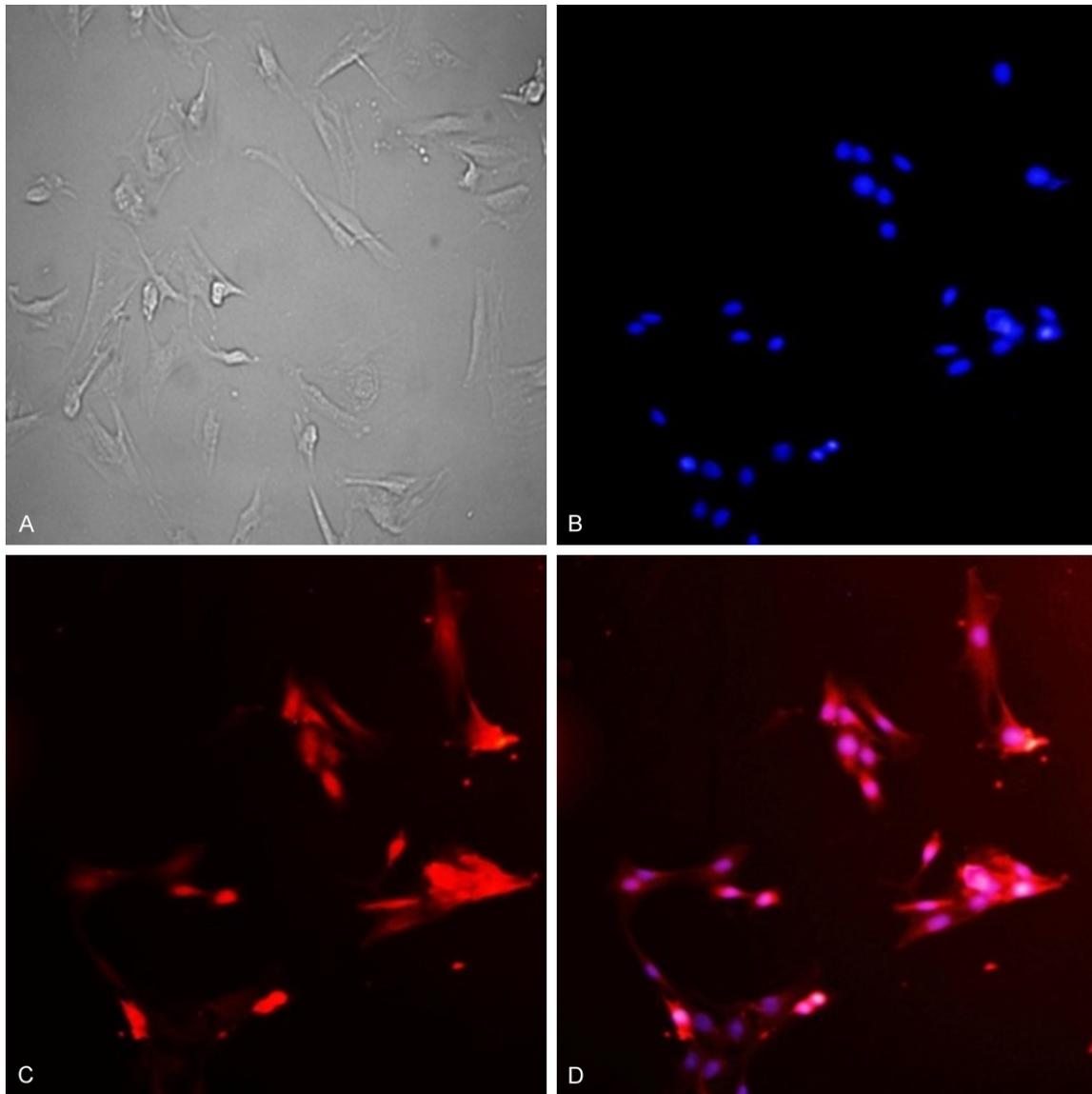


Figure 3. Colonic smooth muscle cells were successfully identified using immunofluorescence staining using antibody to α -actin ($\times 200$). A. SMCs. B. Hoechst 33258 staining (blue). C. α -actin staining (red). D. Merged images.

smooth muscle strips, and this effect could be partially by chelerythrine; and (2) AGEs decreased intracellular calcium concentration, via regulation of the PKC-InsP₃R3 signal transduction pathway. AGEs appear to activate the PKC pathway and thereby increase the phosphorylation of InsP₃R3, which regulates [Ca²⁺]_i. The activity of PKC has been shown to enhance I_{Ca-L} in smooth muscle cells [12]. For example, cholecystokinin increases the I_{Ca-L} of the proximal colon in guinea pigs via the PKC pathway [13]. In general, PKC is an important kinase for cell proliferation, the regulation of ion channels, and the regulation of movement [14, 15].

Previous studies have shown that some substances can activate phospholipase C PLC, leading to the production of diacylglycerol and inositol 1,4,5-triphosphate (IP₃). IP₃ can combine with endoplasmic reticulum InsP₃R3, causing calcium release from the endoplasmic reticulum. Activation of PKC, however, can negatively regulate calcium release from the endoplasmic reticulum by InsP₃R3 phosphorylation [16-19]. The aim of our study was to investigate whether AGEs can reduce colonic smooth muscle contraction and inhibit calcium release from the endoplasmic reticulum, and the results confirmed this hypothesis. This activity appears

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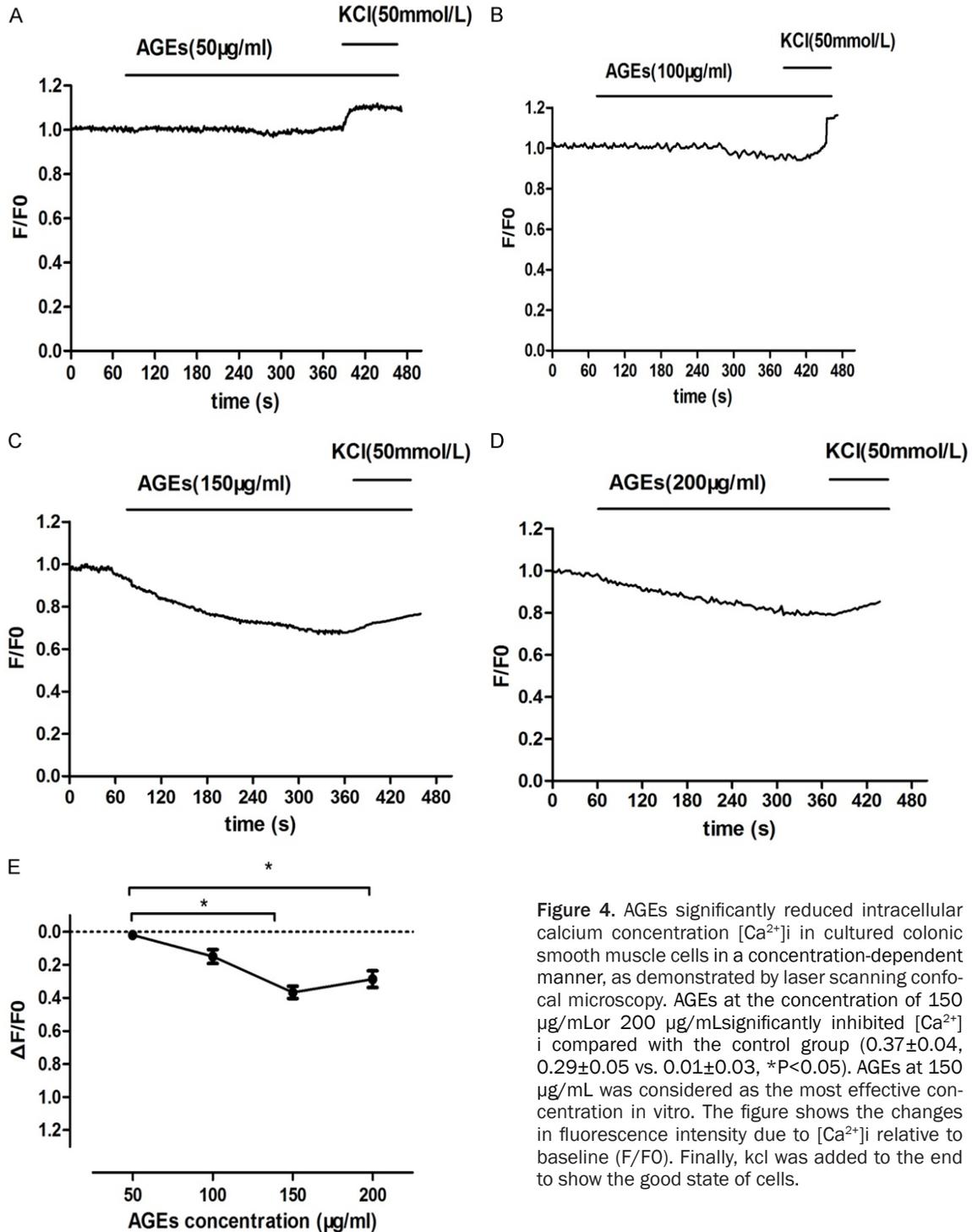


Figure 4. AGEs significantly reduced intracellular calcium concentration $[Ca^{2+}]_i$ in cultured colonic smooth muscle cells in a concentration-dependent manner, as demonstrated by laser scanning confocal microscopy. AGEs at the concentration of 150 $\mu\text{g}/\text{mL}$ or 200 $\mu\text{g}/\text{mL}$ significantly inhibited $[Ca^{2+}]_i$ compared with the control group (0.37 ± 0.04 , 0.29 ± 0.05 vs. 0.01 ± 0.03 , $*P < 0.05$). AGEs at 150 $\mu\text{g}/\text{mL}$ was considered as the most effective concentration in vitro. The figure shows the changes in fluorescence intensity due to $[Ca^{2+}]_i$ relative to baseline (F/F0). Finally, kcl was added to the end to show the good state of cells.

to be mediated through PKC. On one hand, this protective mechanism is induced by excessive release of calcium from the endoplasmic reticulum, and on the other hand functions to prevent excessive release of intracellular calcium that would result in a waste of energy and injury to the cell [20, 21].

Extracellular calcium influx and intracellular calcium release derived from the endoplasmic reticulum are known to be important factors in smooth muscle cell contraction. The increased levels of calcium promote the cross-bridge cycle between actin filaments and myosin heads, resulting in contraction. Then MLCP

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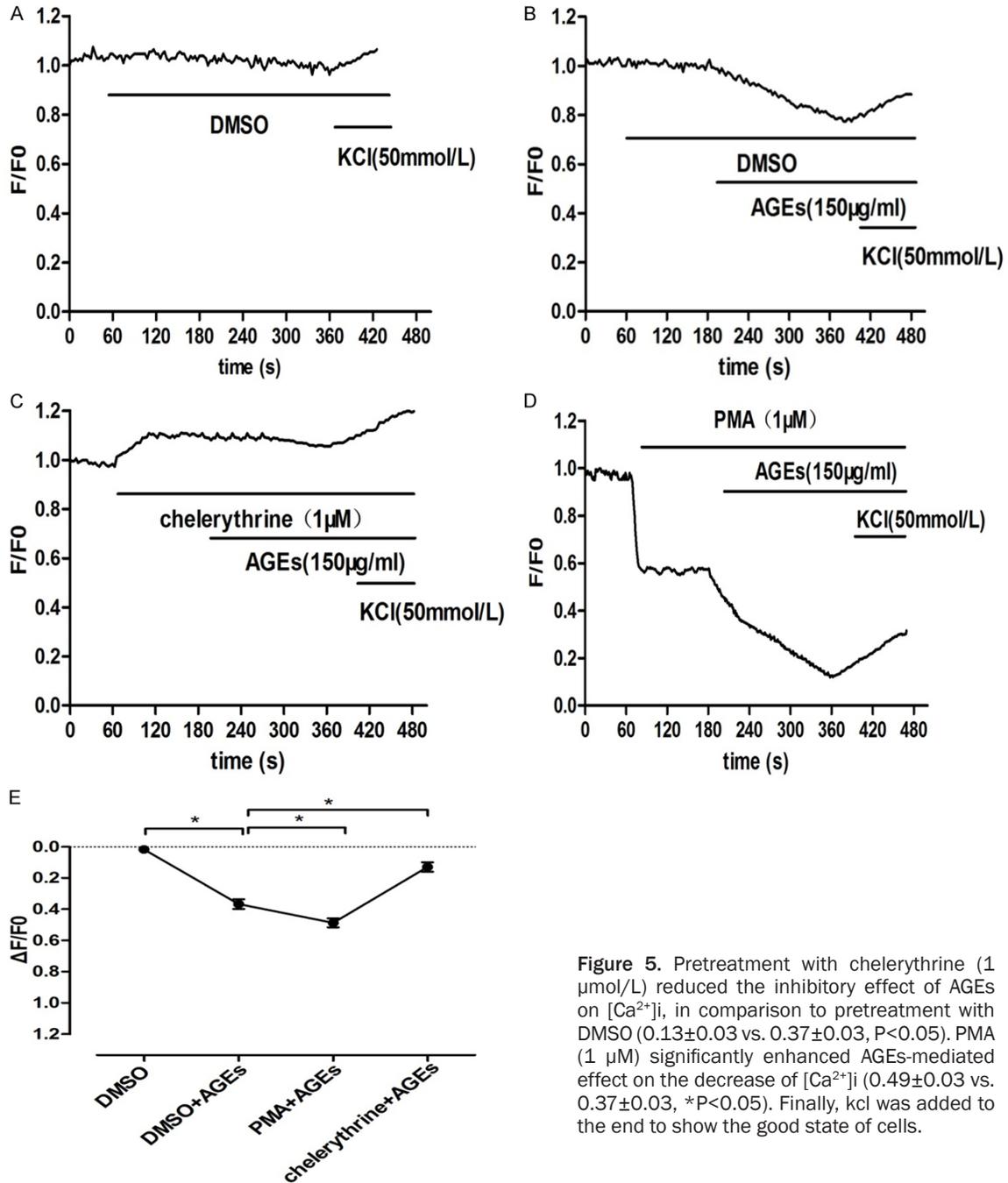


Figure 5. Pretreatment with chelerythrine (1 µmol/L) reduced the inhibitory effect of AGEs on $[Ca^{2+}]_i$, in comparison to pretreatment with DMSO (0.13 ± 0.03 vs. 0.37 ± 0.03 , $P < 0.05$). PMA (1 µM) significantly enhanced AGEs-mediated effect on the decrease of $[Ca^{2+}]_i$ (0.49 ± 0.03 vs. 0.37 ± 0.03 , $*P < 0.05$). Finally, kcl was added to the end to show the good state of cells.

reduces cross-bridge cycling and leads to muscle relaxation [22, 23]. This study aimed to investigate the effect of AGEs on calcium modulation in isolated colonic smooth muscle cell and its possible mechanisms.

Studies have shown that AGEs were significantly increased in serum and various tissues from diabetic patients. AGEs are known to be a risk factor for diabetic complications as a result of

oxidative stress and glycosylation of certain proteins [24-26]. However, the current study is the first to investigate the correlation of AGEs with GI dysfunction in diabetes mellitus. The limitations of this research are firstly that the AGE-related receptors and signaling pathways are still not clear, particularly the receptors on gastrointestinal smooth muscle cell, and so there are no specific receptor antagonists for AGEs [27-29]. The effects of AGEs on intracel-

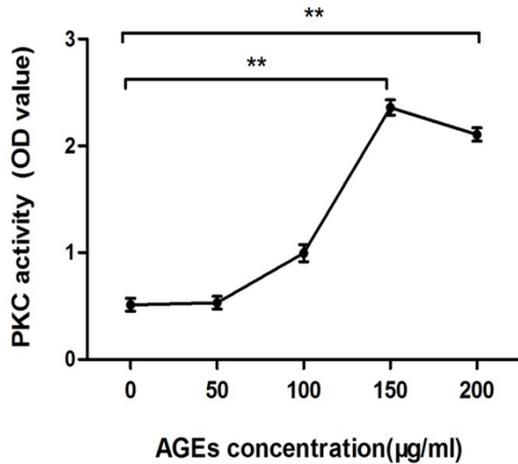


Figure 6. Protein kinase C activity increased in smooth muscle cells treated with 150 µg/mL and 200 µg/mL AGEs compared with the control group (2.36±0.07, 2.10±0.06 vs. 0.51±0.06, **P<0.01).

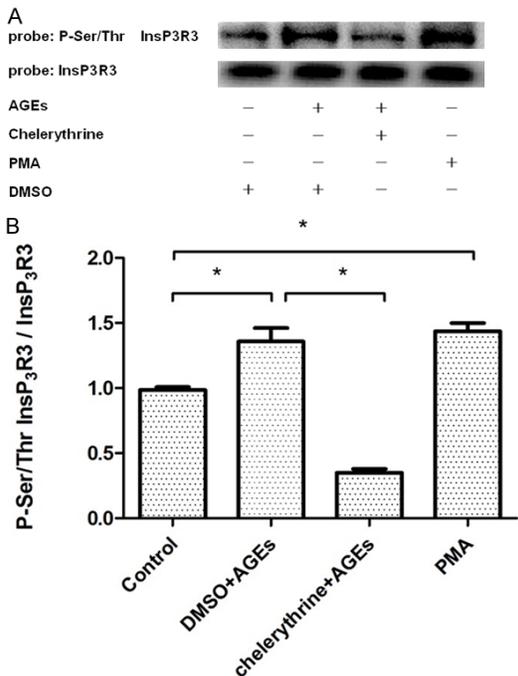


Figure 7. Immunoprecipitation and Western blotting of cultured smooth muscle cells treated with AGEs (150 µg/mL) and controls. AGEs increased the phosphorylation of InsP₃R3, and this effect could be inhibited by chelerythrine (1 µmol/L) (0.34±0.02 vs. 1.35±0.10, *P<0.05). The protein kinase C activator phorbol 12-myristate 13-acetate (PMA; 1 µmol/L), used as a positive control, also enhanced phosphorylation of InsP₃R3 (1.39±0.10 vs. 0.98±0.02, *P<0.05).

lular calcium concentration will be fully explained only when this effect can be blocked by

specific receptor antagonists. Secondly, the possible effects of AGEs on the influx of calcium via extracellular ion channels and the release from the endoplasmic reticulum via other receptors, such as the ryanodine receptor (RyR), need to be further investigated [30-32]. Finally, the intracellular calcium concentration was measured only by laser scanning confocal microscopy. This technique can be combined with other experimental methods such as flow cytometry and the patch clamp technique in for further studies.

Taken together our results demonstrate that AGEs can reduce colonic smooth muscle intracellular calcium concentration, and that this is mediated by PKC-induced phosphorylation of InsP₃R3. AGEs inhibit endoplasmic reticulum calcium release, resulting in a reduction of intracellular calcium. Whether the colonic dysmotility seen in patients with diabetes mellitus is related to increased AGEs, and whether AGEs also inhibit colonic smooth muscle [Ca²⁺]_i via other mechanisms are questions yet to be studied. However, this study suggests a possible mechanism for gastrointestinal dysmotility in diabetes mellitus.

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Disclosure of conflict of interest

None.

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