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Roles of calcium and IP_3 in impaired colon contractility of rats following multiple organ dysfunction syndrome

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Abstract

The purpose of the present study was to explore changes in rat colon motility, and determine the roles of calcium and inositol (1,4,5)-triphosphate (IP_3) in colon dysmotility induced by multiple organ dysfunction syndrome (MODS) caused by bacteria peritonitis. The number of stools, the contractility of the muscle strips and the length of smooth muscle cells (SMC) in the colon, the concentration of calcium and IP_3 in SMC, and serum nitric oxide were measured. Number of stools, fecal weight, IP_3 concentration in SMC and serum nitric oxide concentration were 0.77 ± 0.52 pellets, 2.51 ± 0.39 g, 4.14 ± 2.07 pmol/tube, and 113.95 ± 37.89 μmol/L, respectively, for the MODS group (N = 11) *vs* 1.54 ± 0.64 pellets, 4.32 ± 0.57 g, 8.19 ± 3.11 pmol/tube, and 37.42 ± 19.56 μmol/L for the control group (N = 20; $P < 0.05$). After treatment with 0.1 mM acetylcholine and 0.1 M potassium chloride, the maximum contraction stress of smooth muscle strips, the length of SMC and the changes of calcium concentration were 593 ± 81 and 458 ± 69 g/cm^3, 48.1 ± 11.8 and 69.2 ± 15.7 μM, 250 ± 70 and 167 ± 48%, respectively, for the control group *vs* 321 ± 53 and 284 ± 56 g/cm^3, 65.1 ± 18.5 and 87.2 ± 23.7 μM, 127 ± 35 and 112 ± 35% for the MODS group ($P < 0.05$). Thus, colon contractility was decreased in MODS, a result possibly related to reduced calcium concentration and IP_3 in SMC.

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Introduction

Although multiple organ dysfunction syndrome (MODS) is the most common cause of death in intensive care units, its pathophysiology is incompletely understood. MODS is frequently followed by gastrointestinal stasis, which can increase gut permeability and bacterial translocation. Bacteria and endotoxin from the gut lumen subsequently enter the portal or systemic circulation. In addition, the translocation of bacteria and endotoxin may lead to local inflammation with the production of cytokines and other inflammatory mediators. These intestinally derived mediators may exacerbate the systemic inflammatory response and MODS. A vicious cycle of increased intestinal permeability leading to toxic mediator release results in a further increase in gut permeabil-

ity (1-3). Therefore, it is clear that increased gut permeability and bacterial translocation, which mainly result from gut stasis, play a role in MODS (4-7).

Calcium plays a key role in smooth muscle cell (SMC) contraction. The increase and fall of intracellular free Ca^{2+} initiates contraction and relaxation in smooth muscles (8). Lodato et al. (9) reported that gastrointestinal stasis during sepsis may be associated with gastrointestinal smooth muscle dysfunction. The septic syndrome is a systemic inflammatory response to invading microorganisms. This response is now believed to be the result of a cascade of mediators: microbial toxins, such as lipopolysaccharide endotoxin of Gram-negative bacteria, cause host release of potent proinflammatory mediators, which in turn are largely responsible for cardiovascular derangement, shock, and MODS. MODS is the result of sepsis in experimental animals. Endotoxin impairs smooth muscle contraction, in part by induction of nitric oxide (NO) synthase and enhanced NO production. Decreased ileal muscle contractility has been reported (9), but colon contractility in MODS animals has not been reported. Therefore, we postulated that colon contractility was weakened during MODS, possibly due to changes in calcium, inositol (1,4,5)-triphosphate (IP_3) and NO. The current study was designed to examine the changes in colon contraction and the role of calcium, IP_3 and NO in MODS.

Material and Methods

Material

Male Wistar rats weighing 200-250 g were supplied by the Center of Experimental Animals at Sichuan University. Animals were bred in a controlled environment with a 12-h light/dark cycle. A Biopac system MP150 Physiometer (Biopac, Goleta, CA, USA) was used to measure contractility of circular colon muscle strips. Laser-scanning confocal microscopy (Radiance 2000; Bio-Rad, Hertfordshire, UK) was used to determine the concentration of calcium in SMC. An image analysis system (Cimas 2000, Beijing, China) was used to observe the change in SMC length. Collagenase type II (Sigma, St. Louis, MO, USA), Fluo-3 AM, F-127 (Molecular Probes, Eugene, OR, USA), an IP_3 kit (Amersham Biosciences Inc., Pittsburgh, PA, USA), and an NO test kit were also used in this study.

Methods

Establishment of the MODS model. The study was approved for animal use by Sichuan University and conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals (10,11). Forty male Wistar rats were randomly divided into two groups of 20 animals each. The MODS model was established according to Zheyu (12). Briefly, a 1-mL suspension of 8 x 10^8 CFU/mL O127H6 *Escherichia coli* (Sigma) containing 10% $BaSO_4$ was injected under sterile conditions into the abdominal cavity of the animals in the MODS group at 8:00 am. The control group was injected with 1 mL of normal saline.

Measurement of stool number. The rats in the two groups were housed separately during the 24-h observation period. The filter papers on the floor of the mouse cages were changed hourly and the number of stools was counted.

Immunohistochemistry

Inducible NO synthase (iNOS) immunostaining was performed on colon segments. Specimens were quenched in 3% H_2O_2-methanol for 20 min, washed with 50 mM phosphate-buffered saline (PBS) three times for 5 min each, incubated in 10% blocking serum for 30 min, and then incubated overnight at 4ºC with rabbit polyclonal antihu-

man iNOS antibody, which cross-reacts with rat iNOS (1:50 dilution, Endogen, Cambridge, MA, USA), followed by three 5-min washes in 50 mM PBS. The specimens were then incubated with biotinylated horse anti-rabbit antibody (1:250 dilution; Vector, Burlingame, CA, USA) at 4ºC for 4 h, washed three times in PBS for 5 min each, incubated with the streptavidin-peroxidase complex for 40 min (ABC Elite kit; Vector), washed three times for 5 min each in PBS, and developed with 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.5% Tris buffer for 5 min at room temperature. Specimens were rinsed and mounted in Gel-Mount (Biomeda Corp., Foster City, CA, USA), coverslipped and examined by light microscopy after staining (Nikon FXA, Fryer, Huntley, IL, USA). Antibodies were diluted in 50 mM PBS containing 0.2% bovine serum albumin (Sigma), 100 units/mL penicillin G, and 100 g/mL streptomycin (Boehringer Mannheim, Indianapolis, IN, USA).

Preparation and measurement of smooth muscle strips. Twenty-four hours after injection, the rats were killed, and the distal colon was rapidly removed and placed in cold preoxygenated Krebs-Ringer buffer (Sigma). Standard Krebs-Ringer buffer contained 137.4 mM Na^+, 5.9 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}, 134 mM Cl^-, 15.5 mM HCO_3^-, 1.2 mM $H_2PO_4^-$, and 11.5 mM glucose. This physiological solution was gassed with 97% O_2-3% CO_2 to establish a pH of 7.4. The intestinal mucosa was curetted in order to obtain 15 five-mm circular muscle strips. One end of the strip was tied to a fixed post and the other attached to an isometric force transducer. In the organ chamber, each strip was allowed to equilibrate for at least 20 min, and stimulators were then added. The stimulators included 0.1 mM acetylcholine (Ach) and 0.1 M potassium chloride, whose concentrations were slightly modified according to the literature (13,14). The force of contraction produced by stimulators was recorded at increasing muscle lengths. After contractile activity was recorded, the length of each strip was measured, and each strip was removed, blotted lightly, and weighed. The cross-sectional area of each strip was calculated from length and weight data by assuming that the density of smooth muscle was 1.05 g/cm^3. All force development was normalized for tissue cross-sectional area and is reported as stress (9).

Preparation of dispersed smooth muscle cells. SMC were isolated from the circular muscle layer of the rat colon as described previously, with slight modifications (15). Briefly, muscle strips were digested for 30 min at 31ºC in HEPES buffer, pH 7.4, of the following composition: 24.6 M HEPES, 115 M NaCl, 5.7 M KCl, 2.0 M KH_2PO_4, 1.9 M $CaCl_2$, 0.6 M $MgCl_2$, 5.6 M glucose, and 0.184% (w/v) DMEM and containing 0.1% type II collagenase and 0.01% trypsin inhibitor. The partly digested strips were washed with PBS and muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through a 500-µm Nitex filter and centrifuged at 350 *g* for 10 min. The filtrate (cell suspension) was equilibrated for 20 min before the experiment. For some experiments, cells were permeabilized by a brief exposure to saponin (75 µg/mL for 4 min) and equilibrated in a cytosolic buffer.

Measurement of smooth muscle cell contraction

The contraction of SMC was measured using computerized image micrometry as described previously (16). Briefly, a 0.25-mL cell suspension consisting of 1 x 10^4 cells was added to 0.1 mL of the solution containing the test agents. The reaction was interrupted after 1 min by adding 0.1 mL acrolein at a final concentration of 0.1%. Individual cell length was measured by computerized image micrometry and the average length of cells before and after adding the

test agents was obtained for 50 randomly chosen cells. The contractile response after each agent was defined as the decrease in the average length of the 50 cells and expressed as a percentage of the length before agent administration (17,18).

Effect of L-N6-(1-iminoethyl)-lysine on smooth muscle strips and smooth muscle cells

The contractility of smooth muscle strips and SMC pretreated with 100 µM L-N6-(1-iminoethyl)-lysine (L-NIL) (19,20) was measured as described above.

Measurement of intracellular free Ca^{2+} ($(Ca^{2+})i$) *in smooth muscle cells*. Calcium concentration in the SMC was estimated using the Ca^{2+} indicator Fluo-3 AM and a laser-scanning confocal microscope as described by Jacques et al. (21). Briefly, freshly dissociated SMC were seeded onto glass coverslips and incubated with a Fluo-3 working solution (7.5 µmol Fluo-3 AM and 0.02% Pluronic F-127 dissolved in standard buffer) at 37ºC under an atmosphere of 5% CO_2. After a loading period of 30 min, the cells were washed with PBS to remove extracellular Fluo-3 AM followed by incubation for an additional 20 min. Coverslips mounted on the chamber slide (Molecular Probes) were placed on the plate of the confocal microscope. The fluorescence in the cell was excited at 488 nm by an argon-ion laser, and emission between 515-545 nm was detected with a photomultiplier. The amount of Fluo-3 fluorescence indicating the cytosolic Ca^{2+} concentration was recorded (22).

Determination of smooth muscle IP_3 and serum nitric oxide

One milliliter of a cell suspension containing 5 x 10^9 SMC was place in an Eppendorf tube and the amount of IP_3 was measured by radioimmunoassay (23). Serum NO was estimated using a nitrate reductase method (11).

Statistical analysis

Unless otherwise indicated, data are reported as means ± SEM. Means were compared by ANOVA followed by comparisons of individual pairs of means using the Student *t*-test. The chi-square test was used to determine differences in mortality between groups and correlation analysis was also used in this study. The numbers of stools were compared by non-parametric statistical tests included in the SPSS, version 10.1, statistical analysis program (SPSS Inc., Chicago, IL, USA). The level of significance was set at $P < 0.05$ in all analyses.

Results

The pathobiology of multiple organ dysfunction syndrome in the rat model

Twenty-four hours after injection, 11 rats survived in the MODS group (45% mortality) compared to the control group in which all 20 rats survived (no mortality; $P < 0.01$ compared to the MODS group). Blood was sampled from the caudal vein and serum alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured to determine liver and kidney function using an automatic biochemical analyzer (Hitachi-7110A, Tokyo, Japan). The levels of serum ALT and BUN in the control group (N = 20) were 49.51 ± 10.64 IU/L and 4.79 ± 1.14 M, respectively, whereas the serum ALT and BUN levels in the MODS group (N = 11) were three times higher than the upper limit observed in the control group (173.15 ± 30.97 IU/L and 16.79 ± 1.83 M, respectively). This difference was statistically significant and suggested that the liver and kidney function of the MODS group had been severely damaged. This model of MODS is based on the establishment of bacterial peritonitis, which results in a high-mortality rate. This characteristic makes the model well suited for observing pathological changes

and the effects of treatment (24).

Determination of the effect of multiple organ dysfunction syndrome on stool number

The mean number of stools in the MODS group was 0.77 ± 0.52 per rat within 1 h and 17.48 ± 2.71 within 1 day (24 h) *vs* 1.54 ± 0.64 within 1 h and 36.96 ± 3.82 within 1 day (24 h) in the control group. The mean feces weight per rat for 1 day was 2.51 ± 0.39 in the MODS group *vs* 4.32 ± 0.57 g in the control group. These data showed obvious differences between the two groups.

Inducible nitric oxide synthase production in multiple organ dysfunction syndrome colon

Smooth muscle contractility of the intestine during sepsis or after surgical manipulation is regulated, in part, by iNOS activity (25,26). To test the hypothesis that induced NO contributes to the impairment of smooth muscle function of the colon following MODS we performed immunohistochemical techniques to confirm the finding of iNOS production in the colon in MODS at the protein level. iNOS protein was detected in the external muscularis of MODS colon, with the most prominent staining occurring in the longitudinal layer. Staining for iNOS protein was also observed in the submucosa of MODS colon but was patchier. Little iNOS protein was detected in the colon of the control group (Figure 1A,B).

Measurement of the contractile changes of muscle strips and smooth muscle cells and the effects of L-N6-(1-iminoethyl)-lysine on smooth muscle strips and smooth muscle cells

To determine if muscle dysfunction accompanied MODS, mechanical activity was assessed by measuring circular strip contractions. After treatment of the muscle strips with 0.1 mM Ach, the maximal contractile stress was 593 ± 81 in the control group and 321 ± 53 g/cm^3 in the MODS group ($P < 0.01$). After the addition of 0.1 M potassium chloride, the maximal contractile stress was 458 ± 69 in the control group and 284 ± 56 g/cm^3 in the MODS group ($P < 0.01$).

In the control group, the maximal contractile stress was 598 ± 74 g/cm^3 for the strips pretreated with 100 µM L-NIL followed by treatment with 0.1 mM Ach and 461 ± 77 g/cm^3 for the strips pretreated with 100 µM L-NIL followed by 0.1 M potassium chloride. There were no significant differences in the values obtained before and after using L-NIL ($P > 0.05$). In the MODS group, the maximal contractile stress of strips pretreated with 100 µM L-NIL followed by treatment with 0.1 mM Ach was 498 ± 72 g/cm^3 and the maximal contractile stress of strips submitted to the same pretreatment followed by 0.1 M potassium chloride was 401 ± 57 g/cm^3. There were significant differences in the values obtained before and after using L-NIL ($P < 0.05$; Figure 2).

Circular muscle strips from the colon of

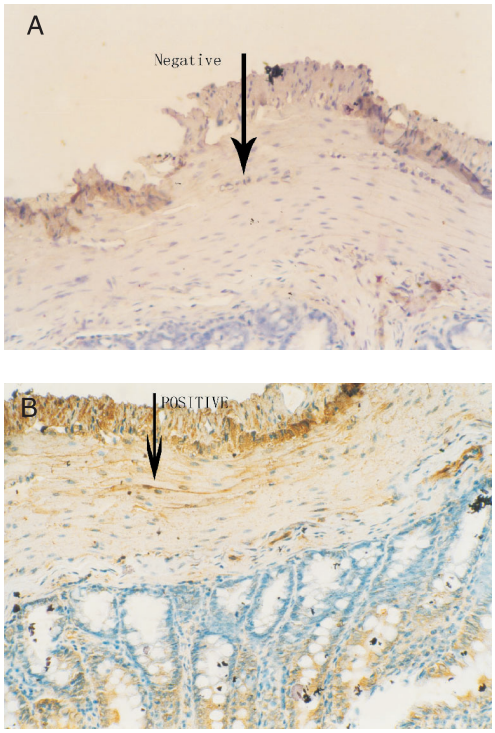

Figure 1. Immunohistochemical analysis of colon sections. Sections showing brown staining were defined as positive. *A*, Negative staining (arrow) for inducible nitric oxide synthase (iNOS) protein in a section from the control group. *B*, Positive staining (arrow) for iNOS protein in a section of the muscularis externa from the multiple organ dysfunction syndrome group (200X).

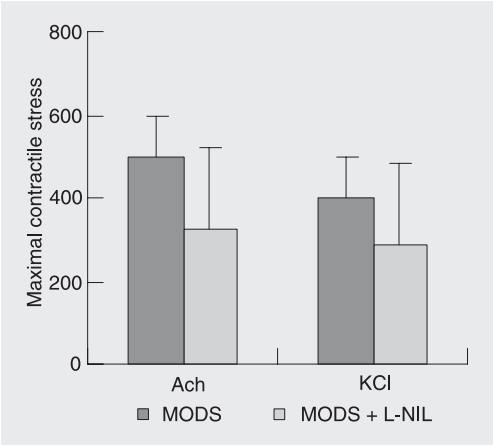

Figure 2. Effect of L-N6-(1-iminoethyl)-lysine (L-NIL) on maximal contractile stress in multiple organ dysfunction syndrome (MODS). In the MODS group, the maximal contractile stress of muscle strips was 321 ± 53 g/cm^3 after treatment with 0.1 mM acetylcholine (Ach), and 498 ± 72 g/cm^3 after strips pretreated with 100 µM L-NIL were treated with 0.1 mM Ach. There were significant differences before and after the use of L-NIL ($P < 0.05$, Student *t*-test). The maximal contractile stress of the muscle strips was 284 ± 56 g/cm^3 after treatment with 0.1 M potassium chloride (KCl) and 401 ± 57 g/cm^3 after strips pretreated with 100 µM L-NIL were treated with 0.1 M KCl. There were significant differences before and after the use of L-NIL ($P < 0.05$, Student *t*-test).

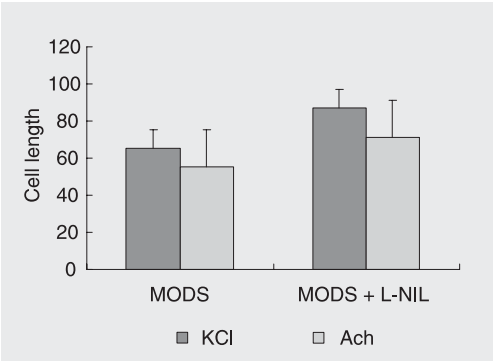

Figure 3. Effect of L-N6-(1-iminoethyl)-lysine (L-NIL) on cell length in multiple organ dysfunction syndrome (MODS). In the MODS group, cell length was 65.12 ± 18.57 and 87.2 ± 23.7 µm after the addition of 0.1 mM acetylcholine (Ach) and 0.1 M potassium chloride (KCl), respectively. When smooth muscle cells (SMC) were pretreated with 100 µM L-NIL, followed by the addition of 0.1 mM Ach and 0.1 M KCl, SMC length was 55.1 ± 9.7 and 71.24 ± 29.4 µm, respectively. There were significant differences in the data before and after the use of L-NIL in the MODS group ($P < 0.05$, Student *t*-test).

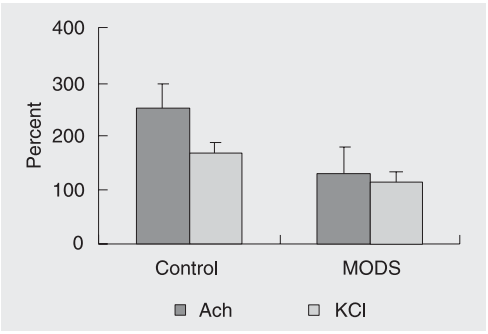

Figure 4. The multiple organ dysfunction syndrome (MODS) group and the control group responded differently in terms of fluorescence intensity of calcium stimulated with 0.1 mM acetylcholine (Ach) and 0.1 M potassium chloride (KCl; $P < 0.05$, Student *t*-test).

control rats generated low-amplitude spontaneous phasic contractions at a frequency of 18.1 ± 0.73 contractions/min. The frequency of spontaneous contractions of colon muscle strips in MODS animals decreased 40% (10.86 ± 0.39 contractions/min) compared to control rats ($P < 0.05$).

In the control group, the length of SMC in the resting state was 75.1 ± 19.8 µm. Following the addition of 0.1 mM Ach and 0.1 M potassium chloride, the length of SMC was 48.1 ± 8.8 and 69.2 ± 15.7 µm, respectively. For the MODS group, the length of SMC in the resting state was 113.2 ± 30.4 µm. Following the addition of 0.1 mM Ach and 0.1 M potassium chloride, the length of SMC was 65.12 ± 18.57 and 87.2 ± 23.7 µm, respectively. The differences in SMC length between groups was significant in both cases ($P < 0.05$).

When SMC were pretreated with 100 µM L-NIL their length in the resting state was 93.1 ± 26.3 µm in the MODS group. Following the addition of 0.1 mM Ach and 0.1 M potassium chloride, the length of SMC was 55.1 ± 9.7 and 71.24 ± 29.4 µm, respectively. The results obtained before and after using L-NIL were significant for the MODS group ($P < 0.05$; Figure 3) but not for the control group ($P > 0.05$).

Determination of the changes of calcium concentration in smooth muscle cells

Treatment with 0.1 mM Ach and 0.1 M potassium chloride led to an increase in the fluorescence intensity measured in the SMC. The average concentrations of calcium ions (fluorescence intensity ratio) in the resting state were 1 ± 0.7 in the SMC of the control group, and 0.7 ± 0.1 in the SMC of the MODS group (12). In the control group, the increase in fluorescence following the addition of 0.1 mM Ach and 0.1 M potassium chloride was 250 ± 70 and 167 ± 48%, respectively, as compared to 127 ± 35 and 112 ± 35% for the MODS group ($P < 0.05$; Figure 4).

Amount of IP_3 in smooth muscle cells and serum nitric oxide concentration

The amount of IP_3 in SMC was 4.14 ± 2.07 pmol/tube in the MODS group *vs* 8.19 ± 3.11 pmol/tube in the control group ($t = 4.78$, $P < 0.05$). The concentration of serum NO was 113.95 ± 37.89 μM in the MODS group *vs* 37.42 ± 19.56 μM in the control group ($t = 7.21$, $P < 0.01$; Tables 1 and 2).

Discussion

The first and optimal pathway for treating gastrointestinal function failure is to restore the motor function of the gut. $(Ca^{2+})i$ plays an important role in regulating contraction and relaxation of smooth muscle. An increase in $(Ca^{2+})i$ triggers contraction in smooth muscle while a fall in $(Ca^{2+})i$ causes relaxation (27,28). In the cytoplasm of SMC, Ca^{2+} binds to calmodulin and the calcium-calmodulin complex combines with the catalytic subunit of myosin light chain (MLC) kinase to form a receptor leading to the phosphorylation of serine at position 19 in the MLC. Phosphorylation of Ser-19 of MLC then allows myosin ATPase to be activated by actin and muscle contraction takes place. When cytoplasmic $(Ca^{2+})i$ is reduced, MLC phosphatase dephosphorylates MLC_{20}, thereby deactivating actomyosin ATPase and causing relaxation (29,30). Ca^{2+} is provided by both extracellular and intracellular sources (31-33).

In the present study, 20 rats in the control group and 11 rats in the MODS group were observed in each contractility study. It was found that the number and weight of feces were lower and the changes of contractile amplitude in the colon smooth muscle strips were less pronounced in the MODS group compared to control. Furthermore, the length of SMC, which represents the contractility of these cells, was greater in the MODS group than in the control group. These results suggest that, after MODS, the motor function of the colon was weakened. In order to study the mechanism of decreased gastrointestinal smooth muscle contraction in MODS, Ach and potassium chloride were used as activators to stimulate SMC contraction. Furthermore, we found that calcium concentration was lower in the MODS group than in the control group, suggesting that colon dysmotility in the MODS group could be related to a fall in calcium concentration. The cause of the decreased calcium concentration in colon SMC in the MODS group was not determined.

The amount of IP_3 in SMC measured by radioimmunoassay was lower in the MODS

Table 1. Correlation between serum nitric oxide (NO) and colon contraction in the multiple organ disfunction syndrome group.

X	Y	r	P
NO	LSMC	0.81	<0.05
NO	MCSSMS	-0.74	<0.05
NO	NS	-0.65	<0.05
NO	WS	-0.68	<0.05

LSMC = length of smooth muscle cells; MCSSMS = maximal contractile stress of smooth muscle strips; NS = number of stools; WS = weight of stools. LSMC, MCSSMS, NS, and WS could show the state of colon contraction. The levels of serum NO had a positive correlation with LSMC, and a negative correlation with MCSSMS, NS, and WS ($P < 0.05$, correlation analysis).

Table 2. Correlation between smooth muscle cell inositol (1,4,5)-triphosphate (IP_3) and colon contraction in the multiple organ disfunction syndrome group.

X	Y	r	P
IP_3	LSMC	0.85	<0.05
IP_3	MCSSMS	-0.71	<0.05
IP_3	NS	-0.69	<0.05
IP_3	WS	-0.64	<0.05

The IP_3 levels of smooth muscle cells had a positive correlation with LSMC, and a negative correlation with MCSSMS, NS, and WS ($P < 0.05$, correlation analysis). For abbreviations, see legend to Table 1.

group than in the control group. A possible conclusion would be that the fall of calcium concentration correlated with the decreased IP_3 in colon SMC in the MODS group. IP_3 is a second messenger in the receptor-G protein-inositol lipid signal transduction pathway, which specifically binds to IP_3 receptors in the sarcoplasmic reticulum. The IP_3 receptors consists of four identical 260-kDa subunits joined by a non-covalent bond. When 3-4 sites are bound by IP_3, the conformation of the receptor complex is altered, the ionic channel is opened and Ca^{2+} is released from intracellular stores (34-36). Therefore, only when the amount of IP_3 in SMC is reduced below a certain threshold is the calcium concentration reduced. This is a potential mechanism of impaired colon contractility in MODS.

By immunohistochemical methods the expression of iNOS was observed in the colon of the MODS group but not in the colon of the control group. Furthermore, contractility was enhanced in the muscle strips and SMC treated with L-NIL, a selective iNOS antagonist. iNOS can produce excessive NO which is the important factor resulting in gastrointestinal stasis and in increased cGMP concentrations in the effector cells (37,38). cGMP can activate protein kinase G and protein kinase A in the cell plasma, causing SMC hyperpolarization, a reduction of Ca^{2+} influx, and an increase in Ca^{2+} uptake by the sarcoplasmic reticulum. Thus, smooth muscle is relaxed and gastrointestinal stasis suffers further deterioration (8,39,40). In the present experiment, the concentration of serum NO was higher in the MODS group than in the control group. Of course, increased serum NO was related to multiple organ dysfunction including circulatory, liver, renal, and respiratory dysfunction, as previously reported (41-43). However, the relation between serum NO and colon contraction in MODS was not mentioned in the cited studies. Therefore, our results only suggest that the reduction of colon contraction in the MODS group could be related to serum NO concentration.

MODS can cause a reduction of gastrointestinal contraction, which may be related to the fall of calcium concentration and IP_3 in SMC. Subsequently, the cGMP and inositol lipid signal transduction pathways are activated simultaneously, leading to the decrease of $(Ca^{2+})i$ in SMC. Toxins and cytokines released during sepsis and MODS also unbalance hemodynamic parameters which, in turn, could induce gastrointestinal dysmotility. These factors should be considered when assessing colon motility, but they were not considered in the present study (6,9).

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