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ORIGINAL ARTICLE

Berberine Depresses Contraction of Smooth Muscle via Inhibiting Myosin Light-chain Kinase

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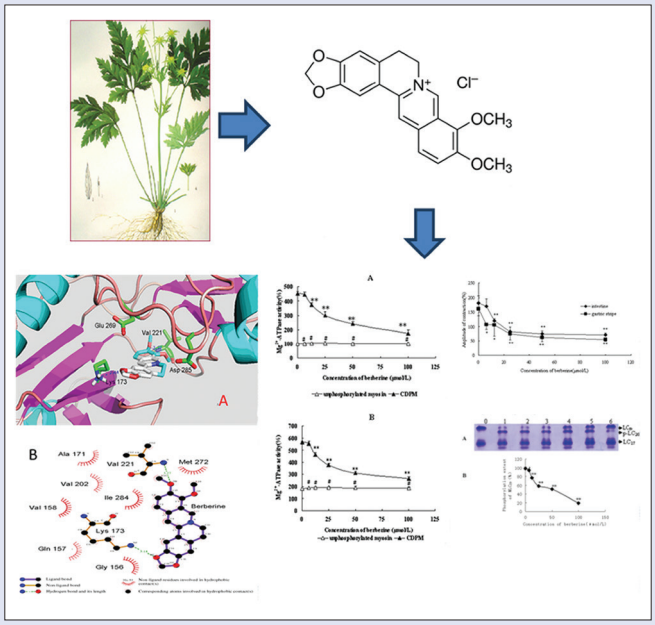
ABSTRACT

Background: Berberine is a natural isoquinoline alkaloid possessing various pharmacological effects, particularly apparent in the treatment of diarrhea, but the underlying mechanism remains unclear. Smooth muscle myosin light-chain kinase (MLCK) plays a crucial role in the smooth muscle relaxation-contraction events, and it is well known that berberine can effectively depress the contraction of smooth muscle. Hence, whether berberine could inhibit MLCK and then depress the smooth muscle contractility might be researched. **Objective:** The purpose of this study is to investigate the effects of berberine on MLCK. Based on this, the contractility of gastro-intestine, catalysis activity of MLCK, and molecular docking are going to be evaluated. **Materials and Methods:** The experiment of smooth muscle contraction was directly monitored the contractions of the isolated gastrointestine by frequency and amplitude at different concentration of berberine. The effects of berberine on MLCK were measured in the presence of Ca^{2+}-calmodulin, using the activities of 20 kDa myosin light chain (MLC_{20}) phosphorylation, and myosin Mg^{2+}-ATPase induced by MLCK. The docking study was conducted with expert software in the meantime. **Results:** The phosphorylation of myosin and the Mg^{2+}-ATPase activity is reduced in the presence of berberine. Moreover, berberine could inhibit the contractibility of isolated gastric intestine smooth muscle. Berberine could bind to the ATP binding site of MLCK through hydrophobic effect and hydrogen bonding according to the docking study. **Conclusion:** The present work gives a deep insight into the molecular mechanism for the treatment of diarrhea with berberine, i.e., berberine could suppress the contractility of smooth muscle through binding to MLCK and depressing the catalysis activity of MLCK.

Key words: Berberine, contraction of smooth muscle, docking study, myosin light-chain kinase

SUMMARY

- Berberine significantly reduced the amplitude of contraction in isolated duodenum and gastric strips in rats
- Berberine inhibited the phosphorylated extents of MLC20 and Mg^{2+}-ATPase activity of phosphorylated myosin induced by MLCK
- Berberine binds to the ATP binding site of MLCK by hydrophobic effect and hydrogen bonding
- Berberine may modulate contraction of smooth muscle by inhibiting MLCK.



Abbreviations used: MLCK: Myosin light chain kinase; MLC_{20}: 20 KDa regulating myosin light chain; CaM: Calmodulin.

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INTRODUCTION

Berberine is an isoquinoline alkaloid present in a number of clinically important medicinal plants, such as *Hydrastis canadensis* (Goldenseal), *Cortex phellodendri* (Huangbai), and *Rhizoma coptidis* (Huanglian), which has a history of usage in Ayurvedic and Chinese medicine dating back at least 3000 years.[1] Modern pharmacological studies showed that berberine holds promising properties as a drug for cardiovascular diseases, diabetes, hyperlipidemia, cancer, diarrhea, Alzheimer's, etc.[2,3] Clinically, berberine has been widely used as antidiarrheal medication. A clinical trial showed that berberine can considerably reduce the volume and frequency of diarrheal stools.[4] An experimental study demonstrated that berberine may exert its antidiarrheal function partially through an antimotility action on intestinal smooth muscle.[4]

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However, the detailed mechanism, especially the impact mechanism of berberine on the catalytic ability of smooth muscle myosin light-chain kinase (MLCK) remains unclear.

MLCK plays a crucial role in the smooth muscle relaxation-contraction events,[5,6] which are regulated by calmodulin (CaM) in the following procedure. At first, the binding of Ca^{2+} to CaM causes CaM to associate with MLCK, which changes the conformation of MLCK from inactive state to active state. Next, the activated MLCK catalyzes phosphorylation of 20 kDa regulatory light chain of myosin (MLC_{20}). Finally, MLC_{20} phosphorylation triggers cycling of myosin crossbridges along with actin filaments, producing a motive force.[7] As a result, inhibiting MLCK can effectively depress the smooth muscle contraction.

In the present work, we showed that berberine can depress the contraction of isolated smooth muscle for latest to corroborate the inhibitory effects on gastrointestinal function. At the same time, we found that the presence of berberine reduced the phosphorylation of MLC_{20} and weakened the activity of Mg^{2+}-ATPase. Both events are dependent on the catalytic function of MLCK. This implies that berberine might directly inhibit MLCK. This hypothesis was tested by a further docking study. Hence, our work could demonstrate that berberine may be a potent MLCK inhibitor, and could terminate the Ca^{2+} signal transduction, resulting in the depression of the smooth muscle contraction.

MATERIALS AND METHODS

Animals, reagents, and other materials

Experiments were performed according to the regulations of animals care with the approval of the Local Animal Protection Committee (Liaoning University of Traditional Chinese Medicine). Male Sprague–Dawley rats (200–250 g) were used to study the effects of berberine on the amplitude of smooth muscle contraction. Animals were purchased from the Experimental Animal Center of Dalian Medical University (Certificate of Conformity: No. SCXK (Liao) 2008-0002). Male Sprague–Dawley rats were housed three per cage. Animals were maintained in a controlled environment at 25°C ± 2°C under a 12 h dark-light cycle (light on at 7:00 A.M.) and acclimatized for at least 5 days before use.

Phenylmethylsulfonyl fluoride and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene glycol-bis (2-aminoethylether) tetraacetic acid was purchased from Wako (Osaka, Japan). Berberine chloride was purchased from Sigma-Aldrich (CAS Number: 633-65-8). All other chemicals were used at the purest commercially available grade.

Krebs's solution was created with the following proportions of constituents (in mmol/L): Sodium chloride 114.0, potassium chloride 4.7, magnesium chloride 1.2, calcium chloride 2.5, sodium dehydrogenate phosphate 1.8, glucose 11.5, and sodium bicarbonate 18.0; pH 7.4.

Preparation of isolated intestine and gastric segments and gastrointestinal motility

The abdomen of rats was opened under urethane anesthesia. The duodenum between the pylorus and the Treitz ligament was removed as described earlier,[8,9] and the duodenum strips were cut in approximate 2 cm segments in length (tubes) to test the effect of berberine on the contractility of the intestinal smooth muscle in rats. The gastric muscle stripes (2 cm long, 0.4 cm wide on average) were obtained as described by others[10,11] and maintained in ice-cold oxygenated Krebs' solution. The mucosa and submucosa were removed gently with fine tweezers. The intestine and gastric muscle stripe were suspended, respectively, in 20 ml chambers containing 37°C Kreb's solution with 95% O_2 and 5% CO_2, equilibrated for 50 min, and the bath solution was replaced every 10 min.

The amplitude (increase in pressure) of the proximal duodenum and gastric stripe contractions was directly monitored using a BL-420F physiological recording system. Occasional movement artifacts were easily identified as spikes that appeared simultaneously in both recorded channels and were eliminated from data analysis. Each animal served as its own control, and recording was limited to 6 h. The effects of the duodenum or the gastric stripe on the isometric response included the relative value (the average of the contractile curve recorded over 5 min) compared to a normal control (Krebs' solution without berberine), which was assigned a value of 100%.

Protein purification

Myosin and MLCK used in the assay were purified from fresh chicken gizzard smooth muscle using the previously reported methods.[12] The actin was purified from acetone powder of chicken breast muscle.[12] The purified myosin was unphosphorylated, which was confirmed by 10% glycerol electrophoresis.

Myosin light chain phosphorylation by myosin light-chain kinase determination

Ca^{2+}-CaM-dependent phosphorylation of MLC by MLCK was carried out according to the method reported previously.[13] After phosphorylation of MLC_{20}, solid urea and sample solution, which contained bromophenol blue and glycerol, were added to the reaction mixture. Next, 10% glycerol polyacrylamide gel electrophoresis was used to measure the extent of phosphorylation of MLC_{20}. Scion Image Software (Frederick, Maryland, USA), densitometry software from Scion Co., Ltd., was applied to analyze the percentage of phosphorylated MLC_{20} in the total MLC_{20}. Monophosphorylation of the positive control was chosen as the control and calculated as 100%.

Measurement of myosin Mg^{2+}-ATPase activity

The method for measuring Mg^{2+}-ATPase activity of myosin was the same as described previously.[14] Briefly, the measuring of Mg^{2+}-ATPase activity was carried out in a 20 mmol/L Tris-HCl (pH 7.4) buffer containing 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.5 mmol/L ATP, 0.1 mmol/L $CaCl_2$, 0.6 mmol/L CaM, and 0.4 μmol/L myosin at 25°C using the malachite green method.

Model building and molecular docking

The homology mode of MLCK of smooth muscle (family number 1) was built with Modeller 9.14[15] using the crystal structure of MLCK (family number 4; PDB code: 2 × 4F) as the template. The docking study was conducted with AutoDock Vina.[16] The center and size of grid box were determined by reference to the position of ligand involved in the structure of 2 × 4F. To fully consider the conformational flexibility, residues involved in the grid box are set to be flexible, while the remaining residues are kept rigid. The predicted conformation with the lowest binding free energy was chosen as the final result and was further analyzed with LIGPLOT[17] and PyMOL 1.0.[18]

Statistical analysis

The results are expressed as means ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed with one-way ANOVA, using the SPSS software (IBM Corporation, Almon City, New York, USA). Statistical significance was adopted at a level of $P < 0.05$.

RESULTS

Effects of berberine on the amplitude of smooth muscle contraction

The high Ca^{2+} (5.0 mmol/L) Krebs solution was chosen as the representative high contractile state to investigate the inhibitory effect of berberine on smooth muscle more clearly. Incubated in high

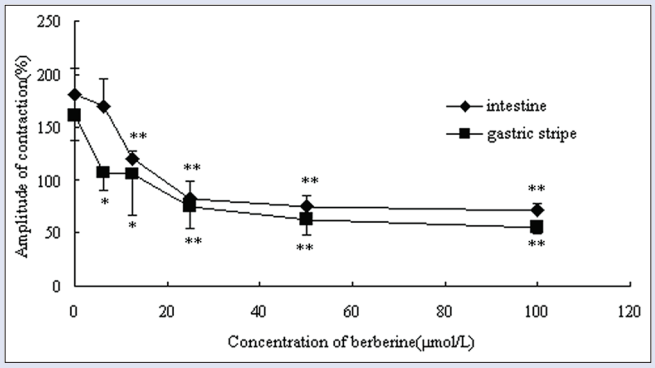

Figure 1: Effect of berberine on the contractility of isolated rat intestine and gastric muscle strips incubated in high Ca^{2+} Krebs buffer ($\bar{x} \pm s$, $n = 6$). *$P < 0.05$, **$P < 0.01$ versus the corresponding controls without berberine

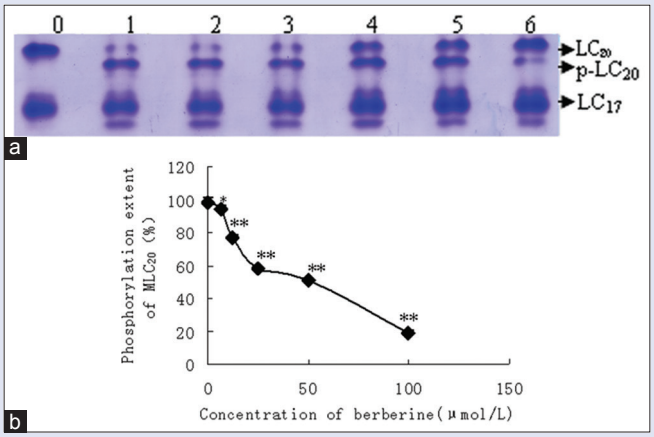

Figure 2: Effects of berberine on phosphorylation of myosin by myosin light-chain kinase ($\bar{x} \pm s$, $n = 6$). The assays are performed in the assay condition described in Materials and Methods. (a) Glycerol electrophoresis results of 20 kDa myosin light chain of myosin phosphorylated by myosin light-chain kinase, LC_{20}, unphosphorylated 20 kDa myosin light chain; p-LC_{20}, monophosphorylated 20 kDa regulating myosin light chain; LC_{17}, 17 KDa myosin essential light chains. Lane 0 is unphosphorylation control, lane 1 is phosphorylation of myosin without berberine administrated, lane 2–6 represent phosphorylation of myosin after 6.25, 12.5, 25, 50, 100 μmol/L berberine administrated. (b) The extent of myosin phosphorylation, which is analyzed using Scion Image Software. Monophosphorylation is chosen as the control and calculated as 100%, and other data are the relative values compared to the control. *$P < 0.05$, **$P < 0.01$ versus phosphorylation without berberine

Ca^{2+} Krebs buffer, both intestinal smooth muscle, and gastric strips showed higher contracted amplitude than that in normal Krebs's solution, respectively. And then, berberine (6.25, 12.5, 25, 50, and 100 μmol/L, respectively) resulted in an inhibition of the amplitude of contraction (from 180.7% ± 25.6% to 170.0% ± 25.6%, 121.3% ± 7.0%, 74.7% ± 21.0%, 72.4% ± 10.0%, and 71.1% ± 6.7% in the duodenum and from 161.1% ±43.5% to 107.4% ±17.6%, 105.7% ± 39.4%, 82.6% ± 16.6%, 72.2% ± 14.4%, and 66.0% ± 6.2% in gastric strips, respectively) in a dose-dependent manner. There was significant difference in the amplitudes between the different concentration groups (12.5 μmol/L, 25 μmol/L, 50 μmol/L, and 100 μmol/L) and the corresponding negative control in intestinal smooth muscle, (**$P < 0.01$). Moreover, in the case of gastric smooth muscle, each concentration of berberine showed apparent inhibition effect on the contraction [Figure 1].

Effect of berberine on 20 kDa myosin light chain phosphorylation by myosin light-chain kinase

Figure 2 shows that MLC_{20} phosphorylation by MLCK significantly decreased (*$P < 0.05$, **$P < 0.01$) as concentration of berberine increases from 6.25 to 100 μmol/L, indicating that berberine induces a dose-dependent inhibition of MLC_{20} phosphorylation by MLCK.

Effect of berberine on myosin Mg^{2+}-ATPase activity

The results in Figure 3a indicate that in the absence of actin, berberine in the range from 6.25 to 100 μmol/L reduced Mg^{2+}-ATPase activities of phosphorylated myosin from 452.5% to 173.3%, but did not apparently increase the percentage of unphosphorylated myosin ($P > 0.05$). In the presence of actin [Figure 3b], the effects of berberine on Mg^{2+}-ATPase activities of different states of myosin are consistent with those without actin.

Docking berberine into the ATP binding site of myosin light-chain kinase

Our experimental results showed that the presence of berberine depresses the phosphorylation of myosin which is dependent on the catalyzation of MLCK. This implies that berberine may be capable of inhibiting MLCK. Hence, we docked the berberine to the ATP binding site of MLCK to test the hypothesis. Docking study showed that the berberine can bind to the ATP binding site of MLCK with a binding energy of −9.6 Kcal/mol. The binding mode is shown in Figure 4a. Further analysis showed berberine exhibited hydrophobic interaction with several hydrophobic residues including Ala171, Met272, Val202, Ile284, and Val158 present in the binding site [Figure 4b]. This is reasonable considering the hydrophobic character of berberine. In addition, two oxygen atoms from the ether group locating at two ends of berberine form two hydrogen bonds with the hydrogen atoms from $-NH_3^+$ group in the side chain of Lys173 and amide group from the backbone of Val221, respectively. It worth noting that the negatively charged side chains of Glu269 and Asp285 can make the binding of positively charged berberine energetically favorable [Figure 4a], due to the electrostatic attraction between them.

DISCUSSION

Smooth muscle contraction is activated primarily through Ca^{2+}-CaM-dependent phosphorylation of MLC_{20} by MLCK. The phosphorylation can be simply described as the following. Initially, the interaction of Ca^{2+} with CaM induces a conformational change of MLCK, thereby activates MLCK. Then, the activated MLCK catalyzes phosphorylation of MLC_{20}. Finally, MLC_{20} phosphorylation triggers cycling of myosin crossbridges along with actin filaments, resulting in motive force.[6,7] To reveal the underlying mechanism of how berberine affects the contraction of smooth muscle, we investigate the effect of berberine on the activity of MLCK, which is a key enzyme involved in the signal pathway related to smooth muscle contraction. Since both phosphorylation level and Mg^{2+}-ATPase activity of the myosin are dependent on catalyzation of MLCK, they may be used as a reliable index for measuring the activity of MLCK. Surely, effect of reagents on enzymatic activity of MLCK could be induced by mediating the expression level of MLCK protein. Hence, we investigate the berberine with different concentration on the phosphorylated extents of MLC_{20} and Mg^{2+}-ATPase activity of phosphorylated myosin induced by MLCK, under the condition of fixed MLCK concentration. For refraining from the ambiguity like that, all of the determinations were executed *in vitro* and directly showed the interaction between berberine and MLCK in the mimic physiological environment. Our results showed that berberine could inhibit the phosphorylation of MLC_{20} of myosin by MLCK, and

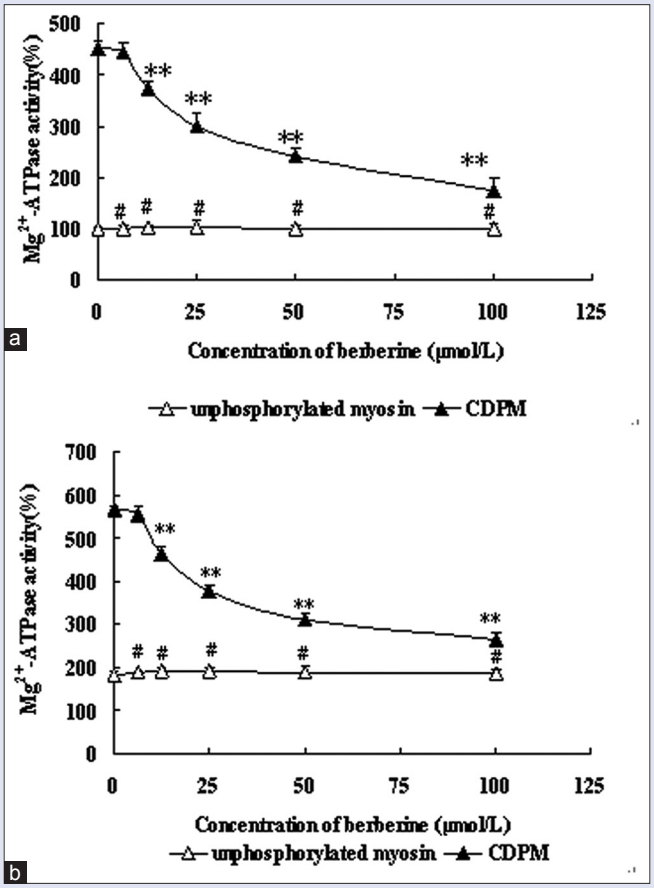

Figure 3: The effect of berberine on Mg^{2+}-ATPase activities of phosphorylated and unphosphorylated myosin ($\bar{x} \pm s$, $n = 6$). (a) Mg^{2+}-ATPase activities in the absence of actin. (b) The assay conducted in the presence of actin. The Mg^{2+}-ATPase activity of unphosphorylated myosin is calculated as 100% in the absence of berberine and actin. $^{**}P < 0.01$, $^{\#}P > 0.05$ versus the corresponding controls without berberine. CDPM, representative Ca^{2+}-CaM-dependent phosphorylated 20 kDa myosin light chain catalyzed by myosin light-chain kinase

reduce the Mg^{2+}-ATPase activity of phosphorylated myosin induced by MLCK. Based on the experimental observation, we propose that berberine may play its role by directly inhibiting MLCK. Our molecular docking study proves that berberine binds to the ATP binding site of MLCK by hydrophobic effect and hydrogen bonding. Hence, based on the discussion above, it can be concluded that berberine may modulate the contraction of smooth muscle by directly inhibiting MLCK.

As is known, diarrhea caused by bacterial infection, inflammatory bowel disease, etc., always involves in the defects of intestinal barrier function, which is critical to the initiation and progression of diarrhea. Moreover, the MLCK is highly expressed following by the defects of intestinal barrier.[19,20] Now that, the inhibitory effects of berberine on smooth muscle contractility are helpful to cure diarrhea clinically, berberine inhibiting MLCK activity might be a deep insight into the molecular mechanism for the treatment of diarrhea with berberine.

CONCLUSION

Berberine could reduce smooth muscle contraction and inhibit the phosphorylation of MLC_{20} and the activity of Mg^{2+}-ATPase of smooth muscle myosin. However, both extent of phosphorylation of MLC_{20} and activity of Mg^{2+}-ATPase are directly related to the activity of MLCK.

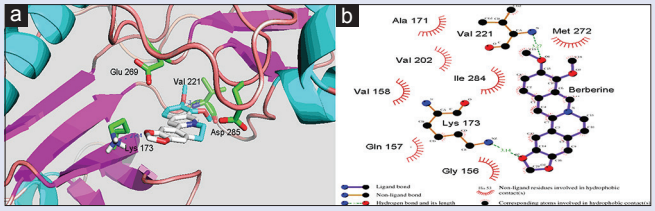

Figure 4: Docking berberine into the ATP binding site of myosin light-chain kinase. (a) The binding mode of berberine to the ATP binding site of myosin light-chain kinase. Berberine forms two hydrogen bonds with Lys173 and Val221. The negatively charged side chains of Asp285 and Glu269 may stabilize the binding of the positively charged berberine via electrostatic attraction. (b) Illustration of interaction between berberine and myosin light-chain kinase. The binding of berberine to the myosin light-chain kinase is driven by the hydrophobic interaction with residues such as Ala171, Met272, Val202, Ile284, Val158, in addition to two hydrogen bonds

This indicates that berberine may play its role by directly inhibiting MLCK by hydrophobic interaction and hydrogen bonding. Hence, berberine may modulate the contraction of smooth muscle by inhibiting MLCK. This finding also provides new clue to other medication effects of berberine,[21] since it may inhibit other kinase to a certain extent due to the structural similarity of the ATP binding site among different kinases.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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