Modulatory Effect of Magnolol in Colonic Motility Dysfunction Induced by Neonatal Maternal Separation in Rats

Zhang Man

A thesis submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Principal Supervisor: Dr. Bian Zhao-Xiang

Hong Kong Baptist University

August 2010
ABSTRACT

Colonic motility dysfunction is one of the main clinical features of irritable bowel syndrome (IBS), which is a common disease of gastrointestinal functional disorders. Stress plays an important role in the onset and development of IBS and can cause the colonic motility dysfunction. The etiology colonic motility dysfunction has not been clarified and current therapies are not perfect. Magnolol, 5,5′-Diallyl-2,2′-biphenyldiol, is a bioactive compound found in the bark of the herb Cortex Magnoliae Officinalis, which has been used for lower gastrointestinal disorders such as diarrhea and constipation in Traditional Chinese medicine (TCM) for long time but the mechanisms have not been well understood. The aims of this study are: 1) to investigate the colonic motility status induced by neonatal maternal separation (NMS); 2) to investigate whether NMS-induced colonic motility dysfunction is related to the change of L-type calcium channels; 3) to investigate the pharmacological effect of magnolol on colonic motility function in NMS rats; and 4) to investigate the possible underlying mechanism of magnolol’s effect.

In in vivo study, the changes of colonic transit were detected through water avoidance stress (WAS) test. In in vitro study, the spontaneous contraction and smooth muscle contractions of colonic smooth muscles under different ligands were recorded through an organ bath system. Laser confocal fluorescent imaging study method was used to compare intracellular calcium concentration ([Ca^{2+}]_i) under different agents in enzymatically isolated single colonic smooth muscle cells of rats. The change of L-type
Ca\textsuperscript{2+} channels currents in the single colonic smooth muscle cells were recorded by using patch clamp technique. Also the immunofluorescence and Western blotting analysis were used to compare the expression of relative proteins.

The results of this study are summarized as follows:

1. NMS increased (1) the fecal pellet number in response to one hour water avoidance stress; (2) the amplitude of spontaneous contraction of colonic sections; (3) contractile responses induced by L-type calcium channels activator Bay K 8644, high K\textsuperscript{+} ions and ACh; and elevated contractile activities was accompanied with an increase of [Ca\textsuperscript{2+}]i in colonic myocytes; (4) the expression of L-type Ca\textsuperscript{2+} channels $\alpha_{1c}$-subunit protein in colonic smooth muscle. These data indicate that NMS induces colonic motility disorder, which is associated with the up-regulation of L-type Ca\textsuperscript{2+} channels in colonic smooth muscles.

2. Magnolol dose-dependently inhibited (1) the colonic motility under one hour WAS \textit{in vivo} in NMS rats; (2) the colonic smooth muscle spontaneous contraction and contractile effect induced by different stimuli \textit{in vitro} in NMS rats; (3) the increased colonic motility pretreatment with Bay K 8644 under 1 hour WAS \textit{in vivo} with nonhandled (NH) rats; (4) [Ca\textsuperscript{2+}]i of colonic smooth muscle cells in NMS rats. These data suggest that magnolol can regulate abnormal colonic motility, and the effect may be associated with the inhibitory effect on activity of voltage-sensitive L-type calcium channels,
3. Magnolol dose-dependently inhibited 1) the spontaneous contractions of colonic smooth muscle and Bay K 8644-, ACh- and KCl-induced contractions in the rat colons in vitro in NH rats; 2) L-type Ca$^{2+}$- channel currents in isolated single colonic smooth muscle cell; 3) expression of $\alpha_{1c}$-subunit of L-type Ca$^{2+}$ channels in rat colonic smooth muscles. These results suggest that magnolol caused the relaxation effect on distal colonic smooth muscle in rat through inhibition of L-type Ca$^{2+}$ channels activities and expression in smooth muscle cells, indicating that the magnolol could act as an L-type calcium channels blocker, and has the potential to be used as an agent for treating IBS.

4. Magnolol at the concentration of 10 µM - 100 µM inhibited smooth muscle contractility in the presence of Ca$^{2+}$ but had no inhibitory effect in the absence of Ca$^{2+}$ after activation of PKC with 30 nM phorbol 12-myristate 13-acetate (PMA). Western blotting results showed that magnolol inhibited the expression of MYL9 (MLC$_{20}$) on smooth muscle but could not affect the expression of MLCK and CPI-17 on smooth muscle. These data suggest that the inhibitory effect of mangolol is involved in the Ca$^{2+}$ dependent PKC pathway. Furthermore, the effect is accompanied by a reduction of MLC$_{20}$ on the colonic smooth muscle.

In conclusion, the results highlight the role of L-type Ca$^{2+}$ channels in the pathophysiological mechanism of NMS-induced colonic motility dysfunction, and clarify that the magnolol can inhibit the colonic muscle contraction; and this effect is mediated through the L-type Ca$^{2+}$ channel signaling. These data indicate that magnolol
could be used as a potential L-type calcium channels blocker to treat IBS.
# TABLE OF CONTENTS

DECLARATION ............................................................................................................... i  
ABSTRACT ...................................................................................................................... ii  
ACKNOWLEDGEMENTS ............................................................................................. vi  
TABLE OF CONTENTS ............................................................................................... viii  
LIST OF TABLES ......................................................................................................... xiii  
LIST OF FIGURES ....................................................................................................... xiv  
LIST OF ABBREVIATIONS ........................................................................................ xvi  
CHAPTER 1 INTRODUCTION ...................................................................................... 1  
  1 Functional gastrointestinal (GI) disorder and motility dysfunction ............... 2  
    1.1 Functional GI disease .................................................................................. 2  
      1.1.1 Functional GI disorders (FGIDs) ..................................................... 2  
      1.1.2 Symptoms induced by GI motility disorders ................................... 4  
  1.2 Irritable bowel syndrome (IBS) .................................................................. 6  
    1.2.1 Epidemiology and categorization .................................................... 6  
    1.2.2 Etiological factors ............................................................................ 8  
      1.2.2.1 Genes .................................................................................... 9  
      1.2.2.2 Psychosocial factors............................................................ 10  
      1.2.2.3 Diet...................................................................................... 10  
      1.2.2.4 GI infection......................................................................... 11  
      1.2.2.5 Visceral hypersensitivity..................................................... 12  
    1.2.2.6 Motility disorder ........................................................................ 15  
      A. Clinical features .............................................................................. 17  
      B. Mechanism study .......................................................................... 18  
  2 IBS and Stress ...................................................................................................... 24  
    2.1 Definition of stress .................................................................................... 24  
    2.2 Stress and IBS ........................................................................................... 24  
    2.3 Stress and visceral sensitivity ................................................................... 26  
    2.4 Stress and GI motility dysfunction ........................................................... 27  
    2.5 Other changes caused by stress................................................................. 29  
    2.6 Animal model of IBS ................................................................................ 30  
      2.6.1 Currently available animal models of IBS ..................................... 30  
      2.6.2 NMS model .................................................................................. 31  
      2.6.3 Current understanding of NMS model........................................... 32  
  3 Calcium and motility ........................................................................................... 33  
    3.1 Calcium and colonic smooth muscle contraction ..................................... 33  
      3.1.1 Calcium dependent initial contraction ........................................... 34  
      3.1.2 Calcium independent sustained contraction .................................. 35  
    3.2 L-type calcium channels and colonic smooth muscle contraction ............ 37  
    3.3 L-type calcium channels blockers and IBS ............................................ 38  
  4 Treatments of IBS ................................................................................................ 39
4.1 Conventional medicine ................................................................. 39
  4.1.1 5-HT receptor modulators ......................................................... 40
  4.1.2 Antispasmodic and anticholinergic agents ............................... 41
  4.1.3 Other drugs ........................................................................... 42
4.2 Chinese medicine ........................................................................ 48
  4.2.1 Herbal formulas ..................................................................... 48
  4.2.2 Acupuncture .......................................................................... 50
5 Magnolol and motility disorder ............................................................ 51
  5.1 Rationale of selection ................................................................ 51
  5.2 The chemical structure of magnolol ............................................ 53
  5.3 The pharmacological activities of magnolol ................................. 54
    5.3.1 Antifungal and antibacterial effect ....................................... 54
    5.3.2 Anti-inflammatory effect ....................................................... 54
    5.3.3 Antioxidant and neuroprotective effect ................................. 55
    5.3.4 Anti-tumor and cancer effects .............................................. 56
    5.3.5 Analgesic effects .................................................................. 56
    5.3.6 Relaxation of smooth muscle effects ..................................... 56
6 Scientific Hypotheses ........................................................................ 58
7 Aims and Design of the research ....................................................... 59
  7.1 Aims and Objectives of the Research ......................................... 59
  7.2 Design of the research ............................................................... 60
8 Technique flowchart ........................................................................ 62
CHAPTER 2 MATERIALS AND METHODS ..................................................... 63
  1 Animals ...................................................................................... 64
  2 Neonatal maternal separation (NMS) .......................................... 64
  3 Water avoidance stress (WAS) ..................................................... 65
  4 Colonic motility tests \textit{in vitro} .................................................... 65
  5 Isolation of smooth muscle cells ................................................ 68
  6 Laser confocal fluorescent imaging ............................................ 68
  7 Immunohistochemistry and Immunofluorescence staining .......... 69
    7.1 Tissue preparation ................................................................. 69
    7.2 Immunohistochemistry (IHC) detection ................................. 70
    7.3 Immunofluorescence (IF) detection ....................................... 71
  8 Western blotting analysis ............................................................ 72
  9 Patch-clamp recording of colonic smooth muscle cells ............... 73
 10 Chemicals and solutions ............................................................. 75
    10.1 Chemicals ............................................................................ 75
    10.2 Composition of Krebs solutions ......................................... 78
    10.3 Solutions for IHC ................................................................. 79
    10.4 Solutions for Western blotting .............................................. 80
    10.5 Solutions for isolating smooth muscle cells ......................... 87
    10.6 Solutions for Patch Clamp .................................................. 88
 11 Antibodies Used in WB and IHC .................................................. 89
12 Statistics ..................................................................................... 90
CHAPTER 3 UP-REGULATION OF L-TYPE CALCIUM CHANNELS IN COLONIC SMOOTH MUSCLE CELLS IS INVOLVED IN COLONIC MOTILITY DYSFUNCTION INDUCED BY NMS ................................................................. 91

1 Introduction ............................................................................................................. 92
2 Material and methods ............................................................................................. 94
   2.1 Experiment design .......................................................................................... 94
   2.2 Drug administration .................................................................................... 95
   2.3 Data analysis and statistics ......................................................................... 96
3 Results .................................................................................................................. 96
   3.1 Effect of NMS on colonic motility ............................................................... 96
   3.2 Effect of NMS on spontaneous contractile activity in vitro ................. 97
   3.3 Effect of NMS on colonic contractile response with tetrodotoxin pre-treatment ................................................................................................................. 97
   3.4 Differences of Bay K 8644-evoked colonic contraction in vitro and [Ca\(^{2+}\)], in isolated single colonic myocytes among NMS and NH rats ........... 97
   3.5 Difference of KCl-evoked colonic contraction in vitro and [Ca\(^{2+}\)], in isolated single colonic myocytes among NMS and NH rats ....................... 98
   3.6 Difference of ACh-evoked colonic contraction in vitro and [Ca\(^{2+}\)], in isolated single colonic myocytes among NMS and NH rats ....................... 98
   3.7 Effect of NMS on colonic contractile response to ACh with nifedipine pretreatment ................................................................................................................. 99
   3.8 Expression of L-type Ca\(^{2+}\) channels \(\alpha_{1c}\)-subunit in colonic smooth muscle............................................................................................................................. 100
4 Discussion .............................................................................................................. 100
5 Conclusion .............................................................................................................. 106

CHAPTER 4 MAGNOLOL REGULATES COLONIC MOTILITY BY INHIBITING L-TYPE CALCIUM CHANNELS IN COLONIC SMOOTH MUSCLE CELLS IN NEONATAL MATERNAL SEPARATION RATS ........................................... 115

1 Introduction .......................................................................................................... 116
2 Materials and methods ......................................................................................... 119
   2.1 Experiment design .................................................................................... 119
   2.2 Drug administration ................................................................................ 121
   2.3 Data analysis and statistics ................................................................... 121
3 Results ................................................................................................................ 122
   3.1 Effect of magnolol on fecal pellets during WAS in NMS rats .................. 122
   3.2 Effects of magnolol on colonic motility during WAS in vivo pretreated with Bay K 8644 in NH rats ......................................................................................... 122
   3.3 Time course of magnolol’s inhibitory effect on colonic smooth muscle contraction .................................................................................................................. 123
   3.4 Dose-dependent inhibitory effect of magnolol on colonic smooth muscle contraction in NMS rats ...................................................................................... 123
   3.5 Inhibitory effect of magnolol on contractions induced by KCl, 5-HT, and substance P ................................................................................................................. 124
   3.6 Effect of magnolol on [Ca\(^{2+}\)], in colonic smooth muscle cells of NMS rats
CHAPTER 5 DOWN-REGULATION OF VOLTAGE-DEPENDENT L-TYPE CA\textsuperscript{2+} CHANNELS OF COLONIC SMOOTH MUSCLE CELLS PARTICIPATES IN THE INHIBITORY EFFECT OF MAGNOLOL ON COLONIC MOTILITY IN RATS

1 Introduction ........................................................................................................ 137

2 Materials and methods ....................................................................................... 137
   2.1 Experiment design ................................................................................ 137
   2.2 Drug administration ........................................................................... 138
   2.3 Data analysis and statistics ..................................................................... 140

3 Results ................................................................................................................ 141
   3.1 Effects of magnolol on spontaneous contraction and KCl- and ACh-induced contractions ................................................................. 141
   3.2 Effects of magnolol on ACh- induced contraction after tetrodotoxin treatment ......................................................................................... 141
   3.3 Effects of magnolol on ACh- induced contraction after L-NAME treatment in NH rats .............................................................................. 141
   3.4 Effects of magnolol on Bay K 8644 - induced colonic smooth muscle contraction ...................................................................................... 142
   3.5 Inhibitory effect of magnolol on L-type Ca\textsuperscript{2+} channels currents ........ 142
   3.6 Inhibitory effect of magnolol on expression of α\textsubscript{1C}-subunit of L-type Ca\textsuperscript{2+} channels in NH rats ................................................................. 143

4 Discussion .......................................................................................................... 143

5 Conclusion ......................................................................................................... 147

CHAPTER 6 MAGNOLOL ATTENUATES CONTRACTION BY DECREASING THE ACTIVITIES OF MLCK/MYOSIN LIGHT CHAIN 20 IN COLONIC SMOOTH MUSCLE CELLS ......................................................................................................... 157

1 Introduction........................................................................................................ 158

2 Materials and methods ....................................................................................... 161
   2.1 Experiment design ................................................................................ 161
   2.2 Drug administration ........................................................................... 162
   2.3 Data analysis and statistics ..................................................................... 163

3 Results ................................................................................................................ 163
   3.1 Effects of magnolol on 10 µM ACh-induced colonic muscle contraction after pretreatment with PKC activator ................................................................. 163
   3.2 Effects of magnolol on 40 mM KCl-induced colonic muscle contraction after pretreatment with PKC activator ................................................................. 163
   3.3 Effects of magnolol on 40 mM KCl-induced colonic muscle contraction after pretreatment with PKC activator in Ca\textsuperscript{2+} free Kreb’s solution............... 164
   3.4 Effects of Y-27632 on 10µM ACh-induced colonic muscle contraction and 40 mM KCl-induced muscle contraction after pretreatment with PKC activator ........................................................................................................ 164
   3.5 Effects of magnolol on the expression of MLCK in the colonic smooth muscle...
muscles.................................................................................................................. 165
3.6 Effects of magnolol on the expression of MYL9 (MLC20) in the colonic smooth muscles................................................................................................................. 165
3.7 Effects of magnolol on the expression of CPI-17 in the colonic smooth muscles.................................................................................................................. 165
4 Discussion........................................................................................................... 166
5 Conclusion .......................................................................................................... 169
CHAPTER 7 CONCLUSION ...................................................................................... 178
1 Summary .......................................................................................................... 179
1.1 The increased colonic motility induced by NMS is associated with up-regulation of L-type Ca\textsuperscript{2+} channels in colonic smooth muscle cells. .... 179
1.2 Magnolol inhibited the colonic motility induced by NMS \textit{in vivo} and \textit{in vitro}. .................................................................................................................... 181
1.3 The inhibitory effect of managolol on smooth muscle contraction is mediated through L-type Ca\textsuperscript{2+} channels. ..................................................... 182
1.4 Magnolol attenuates colonic smooth muscle contraction by decreasing the activity of MLCK/myosin light chain 20 in colonic smooth muscle cells. .. 182
2 Prospects .......................................................................................................... 184
2.1 The linkage between the up-regulation of L-type Ca\textsuperscript{2+} channels in colonic smooth muscle cells and colonic motility disorders ....................... 184
2.2 The effect of magnolol on neurotransmitters involved in colonic motility disorders ................................................................................................. 185
2.3 The effect of magnolol on the PKC pathway.............................................. 185
3 Conclusion ......................................................................................................... 186
REFERENCES ...................................................................................................... 188
PUBLICATIONS.................................................................................................... 215
CURRICULUM VITAE........................................................................................... 217