ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Dec; 22 (12): 1135 - 1142
http://www.chinaphar.com

Magnesium lithospermate B inhibits hypoxia-induced calcium influx and nitric oxide release in endothelial cells[1]

LUO Wei-Bo, WANG Yi-Ping[2] (*Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS Radix Salviae Miltiorrhizae; magnesium lithospermate B; ECV304 cell; anoxia; cell survival; lactate dehydrogenase; calcium; nitric oxide; nitric-oxide synthase

ABSTRACT

AIM: To investigate the inhibitory effect of magnesium lithospermate B (MLB) on hypoxia-induced elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) and nitric oxide (NO) release in endothelial cells. **METHODS**: The cultured human umbilical vein endothelial cells (ECV304) were cultured for 30 min under 95 % N_2 and 5 % CO_2. Cell injury was evaluated by dye exclusion test and lactate dehydrogenase (LDH) assay. $[Ca^{2+}]_i$ was determined by Fura 2-AM. NO content was examined by the NO assay kit. Endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) mRNA expressions were measured by semi-quantitative RT-PCR. **RESULTS**: Cell viability was decreased from (93.0 ± 2.6) % in normoxia to (85.5 ± 2.1) % in hypoxia ($P < 0.01$), and LDH release was increased from (41 ± 28) U/L in normoxia to (141 ± 68) U/L in hypoxia ($P < 0.01$) in ECV304 cultured under calcium conditions. MLB 5 and 10 mg/L improved cell viability and inhibited LDH leakage in ECV304. In addition, hypoxia increased $[Ca^{2+}]_i$, NO release, and eNOS and iNOS mRNA expressions in ECV304 ($P < 0.01$). These increases could be inhibited by MLB 5 and 10 mg/L ($P < 0.01$), but they were unaffected by hypoxia under calcium-free conditions. **CONCLUSION**: MLB attenuates hypoxia-induced cell injury and inhibits hypoxia-induced increases of $[Ca^{2+}]_i$, NO release, and eNOS and iNOS mRNA expressions in ECV304 in Krebs' solution containing calcium. The decreases of NO production and eNOS mRNA expression are possibly associated with inhibition of extracellular calcium influx in MLB-treated ECV304.

INTRODUCTION

Endothelial cells (EC), the essential constituent of vessel, are known to modulate vascular reactivity by releasing some vasoactive substances, especially nitric oxide[1]. Nitric oxide (NO) is an important potent mediator in the cardiovascular system, as well as in nervous and immune system[2-4]. NO is synthesized in the endothelium by two major nitric oxide synthases (NOS), namely eNOS, which is calcium/calmodulin dependent, and iNOS, which is not calcium/calmodulin dependent[5]. NO always acts as the dual molecule in cells. Under normal conditions, it acts as a vasodilator that plays a key role in the regulation of vessel tone; however, under abnormal conditions, it is a principal mediator in various pathological processes, and unbalanced production of NO can cause the endothelial dysfunction, and has the cytotoxic effect on EC[6]. Hypoxia may stimulate EC to release NO, and up-regulate eNOS and iNOS mRNA expressions, which will trigger a series of cellular signal transduction processes[7-9].

Calcium is also an important second mediator in various cells. Intracellular calcium concentration ($[Ca^{2+}]_i$) in EC plays an important role in regulating a variety of cellular functions, such as affecting eNOS mRNA expression[3, 10-11]. The change of $[Ca^{2+}]_i$ is mediated by voltage- or ligand-gated Ca^{2+} channels that regulate Ca^{2+} influx across the plasma membrane and/or by ligand-gated Ca^{2+} channels which control the release of Ca^{2+} from intracellular stores[12]. Hypoxia may stimulate extracellular calcium influx in EC, and hypoxia-

[1] Project supported by the special subject of Bioscience & Biotechnology Research, Chinese Academy of Sciences, No STZ-1-09.
[2] Correspondence to Prof WANG Yi-Ping.
Phn 86-21-6431-1833, ext 308. Fax 86-21-6437-0269.
E-mail ypwang@mail.shcnc.ac.cn
Received 2001-06-12 Accepted 2001-11-02

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Dec; 22 (12)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

induced EC injury is also relevant to an increase in $[Ca^{2+}]_i$[13-14].

Magnesium lithospermate B (MLB), a biologically active compound, is newly extracted from a well-known Chinese herb Radix Salviae Miltiorrhizae. Several investigators have found that MLB could improve renal function via the killikrein-prostaglandin system in rats with renal failure[15]. Kamata *et al* found that MLB could endothelium-dependently relax the noradrenaline (NE)-precontracted aorta[16]. Our studies have shown that MLB has cardioprotective effects against ischemia-reperfusion injury *in vitro* and *in vivo*, and inhibit hypoxia-induced contraction in rabbit aorta with endothelium *in vitro* (data not shown). But until now we have not known about the mechanisms of MLB in the aspects of ischemia therapy. In the present study, we aimed to investigate the effect of MLB on hypoxia-induced cell injury, $[Ca^{2+}]_i$, NO release and eNOS and iNOS mRNA expressions in endothelial cells, in order to explore the mechanisms of the protective effect of MLB on ischemia injury.

MATERIALS AND METHODS

Cell culture ECV304, a human umbilical vein endothelial cell line, was obtained from Cell Bank of Chinese Academy of Sciences. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % bovine calf serum, benzylpenicillin 100 kU/L, streptomycin sulfate 100 kU/L, HEPES 4.7 mmol/L, and sodium bicarbonate 44 mmol/L. The cells were incubated at 37 ℃ in humidified incubator gassed with 5 % CO_2.

Drugs and reagents MLB, a gift from Prof XU Ya-Ming (Department of Phytochemistry, Shanghai Institute of Materia Medica), was a yellow powder, purity >95 %. It was dissolved in dH_2O. Krebs' solution containing calcium (mmol/L): NaCl 120, KCl 5.6, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10, $CaCl_2$ 2.5; calcium-free Krebs' solution (mmol/L): NaCl 120, KCl 5.6, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10, egtazic acid 0.5. DMEM (low glucose), TRIzol and M-MLV reverse transcriptase were purchased from Gibco (NY, USA). Fura 2-AM and BSA were purchased from Sigma (MO, USA). *Taq* polymerase was purchased from Sangon (Shanghai, China). NO and LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were of AR grade.

Hypoxia protocol The resulting subconfluent monolayers of ECV304 were digested with 0.125 % trypsin (w/v) and 0.04 % edetic acid (w/v) in *D*-Hanks' solution, and seeded at 5×10^8/L onto 24-well plates or at 1×10^9/L onto 6-well plates. After overnight, the cells were rinsed twice with *D*-Hanks' solution, and incubated in 1 mL Krebs' solution containing calcium or calcium-free Krebs' solution with 1 % bovine serum albumin (BSA, w/v), respectively. Then the plates were placed at a sealed chamber, and bubbled with 95 % N_2 and 5 % CO_2 at 37 ℃ for 30 min hypoxia.

Cell viability After hypoxia, cell injury was assessed by erythrosine B uptake after incubation with 0.4 % erythrosine B in PBS for 5 min[13]. At least 300 cells were counted for each determination. Cells unable to exclude the colorant were considered to be nonviable. Erythrosine B staining only occurred in irreversibly injured cells.

Assay of lactate dehydrogenase (LDH) After hypoxia, the medium was collected, and the amount of LDH released by cells was determined using the LDH assay kit according to the manufacturer's protocol. The absorbance of samples was read at 440 nm.

Measurement of $[Ca^{2+}]_i$ $[Ca^{2+}]_i$ was measured with Fura 2-acetoxymethyl ester (Fura 2-AM)[17]. The confluent monolayers of ECV304 in 24-well plates were preloaded with Fura 2-AM 250 μmol/L (Sigma) for 30 min at room temperature in dark, then 30 min at 37 ℃ in humidified incubator. The final concentration of Fura 2-AM was 2 μmol/L. Afterwards, cells were gently rinsed three times with *D*-Hanks' solution, and incubated under hypoxia as described above. After 30 min hypoxia, cells were digested with 0.125 % trypsin (w/v) and 0.04 % edetic acid (w/v) in *D*-Hanks' solution, suspended in 1 mL Krebs' solution containing calcium or calcium-free Krebs' solution with 1 % BSA, respectively. The cell suspension was transferred to a quartz cuvette at 37 ℃ on a fluorescence spectrophotometer (HITACHI 650-10 S, Japan), and fluorescence was measured at emission wavelength of 510 nm and excitation wavelength respectively of 340 nm and 380 nm. The fluorescence ratio (F_{340}/F_{380}) was calculated as an indicator of $[Ca^{2+}]_i$.

Assay of nitrite and nitrate After hypoxia, the medium was collected, and the amount of NO

released by cells was determined using the NO assay kit according to the manufacturer's protocol. The method involved measuring the amount of NO metabolites (nitrite and nitrate), which were more stable than NO. The absorbance of samples was read at 550 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) After hypoxia, total RNA from cultured cells was extracted with TRIzol (Life Technologies, Gibco). Total RNA (1 μg) was denatured with oligo-dT_{18} (0.5 g/L) at 70 ℃ for 10 min, and reverse transcription (RT) was carried out at 37 ℃ for 60 min in a final volume of 20 μL, with 50 U RNase inhibitor, 5 × RT buffer [Tris-HCl (pH 8.3) 250 mmol/L, KCl 375 mmol/L, $MgCl_2$ 15 mmol/L, DTT 50 mmol/L], DTT 0.1 mol/L, dNTP 10 mmol/L, and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL). The reaction was terminated by heating at 95 ℃ for 5 min[18].

Polymerase chain reaction amplification was performed with specifically designed PCR primers for eNOS and iNOS. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out on the same samples as a parallel control. The primers were as follows: eNOS, sense 5′-GTG ATG GCG AAG CGA GTG AAG-3′; antisense 5′-CCG AGC CCG AAC ACA CAG AAC-3′[18]; iNOS, sense 5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′; antisense 5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′[19]; and GAPDH, sense 5′-CCA TGG AGA AGG CTG GGG-3′; antisense 5′-CAA AGT TGT CAT GGA TGA CC-3′[20]. A 1/10 volume of the RT reaction was used for PCR with magnesium chloride 1.5 mmol/L, dNTP 10 mmol/L, primer 25 μmol/L, 10 × PCR buffer [Tris-HCl (pH 9.0) 100 mmol/L, KCl 100 mmol/L, $(NH_4)_2SO_4$ 80 mmol/L, NP-40] and 2.5 U *Taq* polymerase in a final volume of 50 μL. The samples were overlaid with 25 μL mineral oil. PCR cycles for GAPDH were performed on the following profile: denaturation at 95 ℃ for 5 min and 94 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s for 25 cycles. Touchdown PCR cycles for eNOS were performed as following: denaturation at 95 ℃ for 30 s and annealing temperature decreasing 1 ℃ every second cycle from 70 ℃ to a touchdown at 60 ℃, then denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 30 s for 30 cycles. PCR cycles for iNOS were performed as following: denaturation at 95 ℃ for 5 min and 94 ℃ for 45 s, annealing at 60 ℃ for 45 s, and extension at 72 ℃ for 2 min for 32 cycles, and an additional 7-min extension at 72 ℃. The PCR products were separated by electrophoresis on 2 % agarose gel containing ethidium bromide and visualized under UV light. Identity was confirmed by a gel documentation system (FR-980), and the values were expressed as ratios of eNOS to GAPDH and iNOS to GAPDH.

Statistical analysis All data were expressed as $x \pm s$, and analyzed by Student's t test. $P < 0.05$ was considered significant.

RESULTS

Effects of MLB on hypoxia-induced cell injury in endothelial cells The influence of MLB on cell viability in normal conditions (air) was first analyzed to determine the *in vitro* toxicity of MLB on EC. The cultured EC on 6-well plates were incubated under normoxia in the presence of different concentrations of MLB from 2.5 to 10.0 mg/L. Cell viability was evaluated by a dye exclusion test. The results showed that MLB did not inhibit EC growth, even at high concentration (10 mg/L), MLB did not interfere with the growth of EC incubated in normal conditions (data not shown).

In order to evaluate the hypoxia-induced cell injury in EC, cell viability was determined by a dye exclusion test and expressed as percentage of erythrosine B uptake after 30 min hypoxia. In Krebs' solution containing calcium, cell viability was reduced to 85.5 % after 30 min hypoxia. Compared with normoxia control (93.0 %), the difference was significant ($P < 0.01$). MLB 5 and 10 mg/L attenuated cell injury induced by hypoxia, and cell viability was increased to 92.7 % and 91.6 % ($P < 0.01$), respectively. But at low concentration of 2.5 mg/L, MLB could not attenuate cell injury induced by hypoxia, and its cell viability was 84 % ($P > 0.05$), which was similar to hypoxia control. In calcium-free Krebs' solution, cell injury greatly increased compared with that in Krebs' solution containing calcium ($P < 0.01$). But the values of each group were similar ($P > 0.05$, Fig 1).

Effects of MLB on hypoxia-induced LDH release in endothelial cells In Krebs' solution containing calcium, hypoxia elicited an increase of LDH release in EC, and its value reached (141 ± 68) U/L ($P < 0.01$), while the value of normoxic cells was (41 ± 28) U/L. MLB at concentrations of 2.5, 5, and 10 mg/L decreased hypoxia-induced LDH release in EC, and the LDH values were (80 ± 20) ($P > 0.05$), (59 ±

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Dec; 22 (12)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

19) ($P < 0.05$), and (45 ± 30) U/L ($P < 0.05$), respectively. In calcium-free Krebs' solution, the amount of LDH release was greatly increased in EC. But hypoxia had no effect on LDH release in EC, and the amount of LDH release of each group was similar ($P > 0.05$, Fig 2).

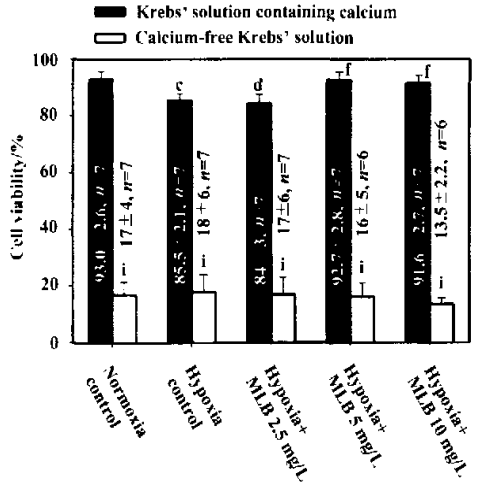

Fig 1. Effect of magnesium lithospermate B (MLB) on hypoxia-induced cell viability in ECV304. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia control. $^dP > 0.05$, $^fP < 0.01$ *vs* hypoxia control. $^iP < 0.01$ *vs* Krebs' solution containing calcium.

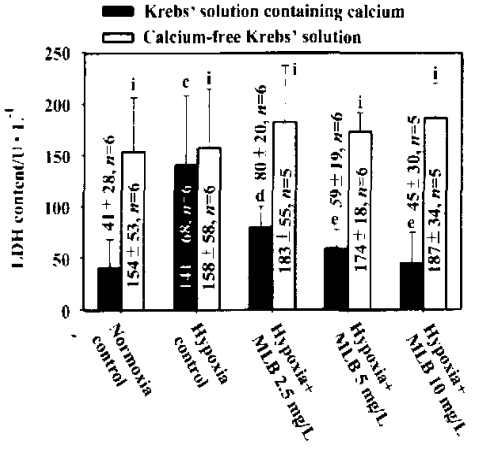

Fig 2. Effect of magnesium lithospermate B (MLB) on hypoxia-induced LDH release in ECV304. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia control. $^dP > 0.05$, $^eP < 0.05$ *vs* hypoxia control. $^iP < 0.01$ *vs* Krebs' solution containing calcium.

Effects of MLB on hypoxia-induced $[Ca^{2+}]_i$ increase in endothelial cells In Krebs' solution containing calcium, the fluorescence ratio (F_{340}/F_{380}) for normoxic cells was 1.85 ± 0.13, while it greatly increased in hypoxia control, and reached 9.1 ± 1.0 ($P < 0.01$). MLB 5 and 10 mg/L reduced hypoxia-induced increase of $[Ca^{2+}]_i$, the F_{340}/F_{380} were 4.8 ± 0.3 and 5.7 ± 1.4 ($P < 0.01$), respectively. But MLB at a concentration of 2.5 mg/L had no effects, and its F_{340}/F_{380} was 9.0 ± 0.9 ($P > 0.05$). In calcium-free Krebs' solution, the fluorescence ratio was decreased compared with that in Krebs' solution containing calcium ($P < 0.01$). Hypoxia could not elicit an increase of $[Ca^{2+}]_i$ in calcium-free Krebs' solution, and MLB at different concentrations of 2.5 to 10 mg/L could not decrease $[Ca^{2+}]_i$ further ($P > 0.05$, Fig 3).

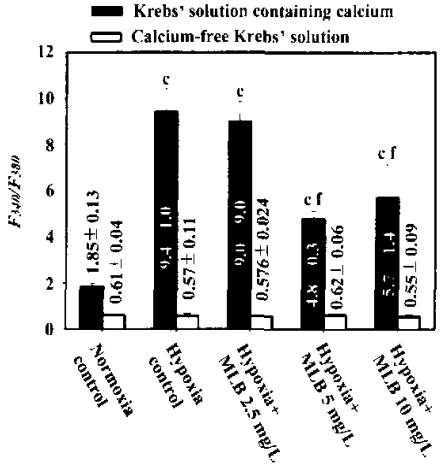

Fig 3. Effect of magnesium lithospermate B (MLB) on hypoxia-induced $[Ca^{2+}]_i$ in ECV304. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia control. $^fP < 0.01$ *vs* hypoxia control.

Effects of MLB on hypoxia-induced NO release in endothelial cells In Krebs' solution containing calcium, hypoxia stimulated NO release in EC. The amount of NO in the culture medium was (17.1 ± 1.2) μmol/L in hypoxia control ($P < 0.01$), while the NO content was (3.3 ± 0.6) μmol/L in normoxia control. MLB 5 and 10 mg/L inhibited hypoxia-induced NO release, and NO contents were (4.8 ± 1.6) and (5.3 ± 0.9) μmol/L ($P < 0.01$), respectively. But MLB 2.5 mg/L had no such effects ($P > 0.05$). In calcium-free Krebs' solution, hypoxia had no effects on NO

release, nor did MLB at different concentrations of 2.5 to 10 mg/L ($P > 0.05$, Fig 4).

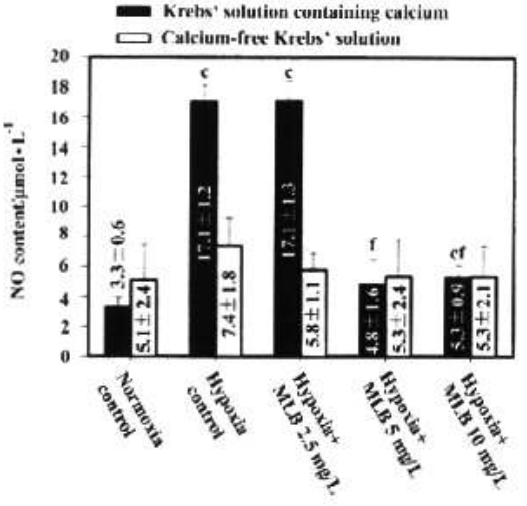

Fig 4. Effect of magnesium lithospermate B (MLB) on hypoxia-induced NO production in ECV304. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia control. $^fP < 0.01$ *vs* hypoxia control.

Effects of MLB on hypoxia-induced eNOS and iNOS mRNA expression in endothelial cells

As shown in Fig 5, in Krebs' solution containing calcium, hypoxia greatly increased the expressions of eNOS and iNOS mRNA compared with normoxia control, and MLB inhibited their expressions in a concentration-dependent manner. In the presence of MLB 5 and 10 mg/L, the ratios of eNOS and iNOS mRNA to GAPDH mRNA were 0.90 ± 0.06 and 0.91 ± 0.08; 0.38 ± 0.03 and 0.35 ± 0.03, respectively, reflecting a significant decrease in levels of mRNA compared with hypoxia control. Compared with Krebs' solution containing calcium, the expression of eNOS mRNA of each group decreased, but the expression of iNOS mRNA increased in calcium-free Krebs' solution. However, the ratios of eNOS and iNOS mRNA to GAPDH mRNA of each group were similar and not significant in calcium-free Krebs' solution (Tab 1).

DISCUSSION

The endothelium plays a key role in regulating vascular homeostasis, due to its unique localization between the intravascular and extravascular spaces. Ischemia is a common clinical situation occurring when blood supply is impaired in a tissue. It has been shown to be rapidly harmful for endothelial cell[14,21-22], and different studies have found a direct link between oxygen lack and disturbance of calcium homeostasis leading to a series of biochemical alterations[23].

In this study, we observed that after 30 min hypoxia, the intracellular calcium concentration of EC cultured in Krebs' solution containing calcium was increased five times more than that in normoxic EC, but

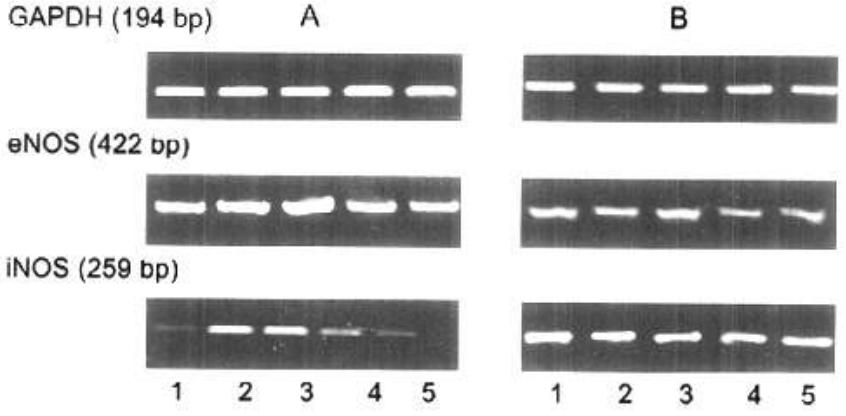

Fig 5. Effect of magnesium lithospermate B (MLB) on hypoxia-induced eNOS and iNOS mRNA expression in ECV304. The expression of eNOS, iNOS and GAPDH mRNA was determined by RT-PCR analysis. PCR products were run and separated by 2 % agarose gel. A: the ECV304 cultured in Krebs' solution containing calcium; B: the ECV304 cultured in calcium-free Krebs' solution. Lane 1: Normoxia control; Lane 2: Hypoxia control; Lane 3: Hypoxia + MLB 2.5 mg/L; Lane 4: Hypoxia + MLB 5 mg/L; Lane 5: Hypoxia + MLB 10 mg/L.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Dec; 22 (12)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Tab 1. Inhibitory effects of magnesium lithospermate B (MLB) on hypoxia-induced eNOS and iNOS mRNA expression in ECV304. The ratios of A_{eNOS}/A_{GAPDH} and A_{iNOS}/A_{GAPDH} were used to express the levels of eNOS and iNOS mRNA in ECV304, respectively. $n = 2$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* normoxia control. $^eP < 0.05$, $^fP < 0.01$ *vs* hypoxia control.

Group	eNOS		iNOS	
	Calcium	Calcium-free	Calcium	Calcium-free
Normoxia control	0.89 ± 0.04	0.76 ± 0.03	0.315 ± 0.021	1.44 ± 0.22
Hypoxia control	1.26 ± 0.06^b	0.75 ± 0.06	1.15 ± 0.04^c	1.48 ± 0.08
MLB 2.5 mg/L	1.21 ± 0.04	0.78 ± 0.04	1.12 ± 0.03	1.46 ± 0.10
MLB 5 mg/L	0.90 ± 0.06^e	0.735 ± 0.021	0.38 ± 0.03^f	1.40 ± 0.22
MLB 10 mg/L	0.91 ± 0.08^e	0.765 ± 0.021	0.35 ± 0.03^f	1.42 ± 0.12

did not increase $[Ca^{2+}]_i$ in calcium-free Krebs' solution, which suggested that hypoxia could induce calcium influx in EC. After MLB treatment, $[Ca^{2+}]_i$ was decreased in EC cultured under calcium conditions. But under calcium-free conditions, MLB had no effects on $[Ca^{2+}]_i$ in hypoxic EC. These results suggested that the reduction of $[Ca^{2+}]_i$ induced by MLB was due to its inhibition on extracellular calcium influx in hypoxic EC. As we know, calcium serves as an important second messenger to elicit a series of signal transductions inside cells. It has been believed that hypoxia can stimulate calcium influx in EC, and the increase in $[Ca^{2+}]_i$ during hypoxic injury is produced as a result of a breakdown in the intracellular calcium homeostasis due to the energy (ATP) depletion, which causes the calcium overload inside cells, and calcium disturbances always trigger cell death[13-14,24].

LDH is located widely in the cytoplasm, and its leakage to extracellular space indicates cell death[25]. Likewise, erythrosine B staining only occurred in injured cells[13]. Erythrosine B uptake and LDH release in hypoxic EC might result from the increase of $[Ca^{2+}]_i$. MLB could attenuate hypoxia-induced cell injury due to its inhibition on extracellular calcium influx. The data of calcium-free Krebs' solution suggested that the reduction of $[Ca^{2+}]_i$ triggered cell death. But MLB could not regulate intracellular calcium homeostasis, so that it could not attenuate cell injury under extracellular calcium-free conditions.

NO is involved in various physiological as well as pathological processes, such as vasorelaxation, inhibition of platelet aggregation, monocyte adhesion to the vessel wall, vascular smooth muscle cell proliferation and migration, as well as atherosclerosis and vasospasm[1,3-4,6,26-29]. However, excess NO production always induces cell injury. In the present work, MLB could decrease NO production, which was favor of reducing cell injury induced by hypoxia.

The major NO is synthesized from *L*-arginine by eNOS in EC. The eNOS is classified as a constitutive and strictly calcium/calmodulin dependent enzyme and its mRNA expression is required for an increase of the intracellular calcium concentration[5,10-11]. Several investigators have indicated that hypoxia could stimulate NO release and eNOS mRNA expression in EC[7-8]. In the present study, we found that with treatment of MLB 5 and 10 mg/L, NO production was decreased and eNOS mRNA expression was inhibited in EC cultured under calcium conditions upon hypoxia, but under calcium-free conditions, they had no such an effect. These results suggested that the inhibitory effect of MLB on NO production and eNOS mRNA expression might result from its inhibition on hypoxia-induced extracellular calcium influx in EC. But MLB at a low concentration of 2.5 mg/L could not inhibit hypoxia-induced extracellular calcium influx, NO content, and eNOS mRNA expression in EC.

iNOS, a calcium-independent NOS isoform, serves as the key role in mediating different aspects of cardiovascular pathophysiology. Its mRNA expression is mainly mediated by several inflammatory and immunological mediators[30]. Ischemia can elicit cells to produce several inflammatory and immunological mediators[31]. It has been believed that hypoxia could induce iNOS gene expression and activate iNOS[9,30]. In the present work, we observed that iNOS mRNA expression increased by 271 % in EC upon hypoxia compared with normoxia, and it was increased under calcium-free conditions compared with that under calcium conditions. MLB 5 and 10 mg/L inhibited iNOS mRNA expression in EC upon hypoxia in the presence of calcium. Our previous study has shown that MLB could

scavenge free radicals and inhibit lipid peroxidation[32]. NO is a potent free radical. It plays the key role in ischemia injury[33]. MLB could decrease NO production which was a result of reduced levels of eNOS and iNOS mRNA, and attenuate free radical injury in EC upon hypoxia.

In conclusion, our results indicated that MLB could inhibit extracellular calcium influx, down-regulate eNOS and iNOS mRNA expressions, decrease NO release, and attenuate cell injury in EC upon hypoxia. It suggests that MLB has protective effect against ischemia at cellular levels.

REFERENCES

1 Furchgott RF, Zawadski JV. The obligatory role of *endothelial cells in the relaxation of arterial smooth muscle by* acetylcholine. Nature 1980; 288: 373 – 6.
2 Shah AM, Vallance P, Harrison D. NO in the cardiovascular system. Cardiovasc Res 1999; 43: 507 – 8.
3 Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 1991; 43: 109 – 42.
4 Bredt DS, Snyder SH. Nitric oxide: A physiologic messenger molecule. Annu Rev Biochem 1994; 63: 175 – 95.
5 Fleming I, Bauersachs J, Busse R. Calcium-dependent and calcium-independent activation of the endothelial NO synthase. J Vasc Res 1997; 34: 165 – 74.
6 Wever RM, Luscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation 1998; 97: 108 – 12.
7 Pohl U, Busse R. *Hypoxia stimulates release of endothelium-derived* relaxant factor. Am J Physiol 1989; 256 (6 Pt 2): H1595 – 600.
8 Arnet UA, McMillan A, Dinerman JL, Ballermann B, Lowenstein CJ. Regulation of endothelial nitric oxide synthase during hypoxia. J Biol Chem 1996; 271: 15069 – 73.
9 Palmer LA, Semenza GL, Stoler MH, Johns RA. Hypoxia induces type Ⅱ NOS gene expression in pulmonary artery endothelial cells via HIF-1. Am J Physiol 1998; 274 (2 Pt 1): L212 – 9.
10 Lin S, Fagan KA, Li KX, Shaul PW, Cooper DM, Rodman DM. Sustained endothelial nitric-oxide synthase activation requires capacitative Ca^{2+} entry. J Biol Chem 2000; 275: 17979 – 85.
11 Wang YP, Shin WS, Kawaguchi H, Inukai M, Kato M, Sakamoto A, *et al*. Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth cells in coculture. J Biol Chem 1996; 271: 5647 – 55.
12 *Adams DJ, Barakeh J, Laskey R, Breemen CV. Ion* channels and regulation of intracellular calcium in vascular endothelial cells. FASEB J 1989; 3: 2389 – 400.
13 Arnould T, Michiels C, Alexandre I, Remacle J. Effect of hypoxia upon intracellular calcium concentration of human endothelial cells. J Cell Physiol 1992; 152: 215 – 21.
14 Aono Y, Ariyoshi H, Sakon M, Ueda A, Tsuji Y, Kawasaki T, *et al*. Human umbilical vein endothelial cells (HUVECs) show Ca^{2+} mobilization as well as Ca^{2+} influx upon hypoxia. J Cell Biochem 2000; 78: 458 – 64.
15 Yokozawa T, Chung HY, Lee TW, Oura H, Nonaka G, *Nishioka I. Magnesium lithospermate B improves renal* function via the kallikrein-prostaglandin system in rats with renal failure. Nippon Jinzo Gakkai Shi 1990; 32: 893 – 8.
16 Kamata K, Iizuka T, Nagai M, Kasuya Y. Endothelium-dependent vasodilator effects of the extract from *Salviae miltiorrhizae* radix. A study on the identification of lithospermic acid B in the extracts. Gen Pharmacol 1993; 24: 977 – 81.
17 Wang YP, Oike M, Ito Y. Effects of superoxide anion in intracellular Ca^{2+} concentration in rabbit pulmonary arterial smooth muscle cells. Acta Pharmacol Sin 1999; 20: 10 – 4.
18 Reiling N, Ulmer AJ, Hauschildt S. Measurement of eNOS and iNOS mRNA expression using reverse transcription polymerase chain reaction. In: Titheradge MA, editor. Methods in molecular biology; v 100. Nitric oxide protocols. *Totona: Humana Press; 1998. p 155 – 61.*
19 Cheung F, Siow YL, Chen WZ, O K. Inhibitory effect of *Ginkgo biloba* extract on the expression of inducible nitric oxide synthase in endothelial cells. Biochem Pharmacol 1999; 58: 1665 – 73.
20 Jiang JF, Liu WJ, Ding J. Regulation of telomerase activity in camptothecin-induced apoptosis of human leukemia HL-60 cells. Acta Pharmacol Sin 2000; 21: 759 – 64.
21 Suval WD, Duran WN, Boric MP, Hobson RW, Berendsen PB, Ritter AB. Microvascular transport and endothelial cell alterations preceding skeletal muscle damage in ischemic and reperfusion injury. Am J Surg 1987; 154: 211 – 8.
22 Forman MB, Puett DW, Virmani R. Endothelial and myocardial injury during ischemia and reperfusion implications. J Am Coll Cardiol 1989; 13: 450 – 9.
23 Piper HM. *Energy deficiency, calcium overload or oxidative* stress: Possible causes of irreversible ischemic myocardial injury. Klin Wochenschr 1989; 67: 465 – 76.
24 Kristian T, Siesjo BK. Calcium in ischemia cell death. Stroke 1998; 29: 705 – 18.
25 Sharikabad MN, Ostbye KM, Lyberg T, Brors O. Effect of extracellular Mg^{2+} on ROS and Ca^{2+} accumulation during reoxygenation of rat. Am J Physiol Heart Circ Physiol 2001; 280: H344 – 53.
26 Wang WZ, Anderson G, Fleming JT, Peter FW, Franken RJ, Acland RD, *et al*. Lack of nitric oxide contributes to vasospasm during ischemia/reperfusion injury. Plast Reconstr Surg 1997; 99: 1099 – 108.
27 Kibbe M, Billiar T, Tzeng E. Inducible nitric oxide synthase and vascular injury. Cardiovasc Res 1999; 43: 650 – 7.
28 Drexler H. *Nitric oxide and coronary endothelial dysfunction* in humans. Cardiovasc Res 1999; 43: 572 – 9.
29 Jeremy JY, Rowe D, Emsley AM, Newby AC. Nitric oxide and the proliferation of vascular smooth cells. Cardiovasc Res

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Dec; 22 (12)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

1999; 43: 580 - 94.
30 Wildhirt SM, Weismueller S, Schulze C, Conrad N, Kornberg A, Reichart B. Inducible nitric oxide synthase activation after ischemia/reperfusion contributes to myocardial dysfunction and extent of infarct size in rabbits: evidence for a late phase of nitric oxide-mediated reperfusion injury. Cardiovasc Res 1999; 43: 698 - 711.
31 Okuma Y, Uehara T, Miyazaki H, Miyasaka T, Nomura Y. The involvement of cytokines, chemokines and inducible nitric oxide synthase (iNOS) induced by a transient ischemia in neuronal survival/death in rat brain. Nippon Yakurigaku Zasshi 1998; 111: 37 - 44.
32 Wu XJ, Wang YP, Wang W, Sun WK, Xu YM, Xuan LJ. Free radical scavenging and inhibition of lipid peroxidation by magnesium lithospermate B. Acta Pharmacol Sin 2000; 21: 855 - 8.
33 Mishra OP, Zanelli S, Ohnishi ST, Delivoria-Papadopoulos M. Hypoxia-induced generation of nitric oxide free radicals in cerebral cortex of newborn guinea pigs. Neurochem Res 2000; 25: 1559 - 65.

丹酚酸 B 镁盐抑制低氧诱导内皮细胞钙内流和一氧化氮释放[1]

罗伟波，王逸平[2] （中国科学院上海生命科学研究院上海药物研究所，上海 200031，中国）

关键词 丹参；丹酚酸 B 镁盐；ECV304 细胞；低氧；细胞存活；乳酸脱氢酶；钙；·氧化氮；·氧化氮合酶

目的：研究丹酚酸 B 镁盐对低氧引起的内皮细胞内钙升高和一氧化氮释放增加的抑制作用. **方法**：培养的人脐静脉内皮细胞暴露在 95 % N_2 + 5 % CO_2 条件下 30 分钟. 内皮细胞的损伤用染料排除实验和 LDH 的释放来评价. 胞内游离钙浓度用钙荧光探针 Fura 2-AM 测定. ·氧化氮含量用一氧化氮试剂盒测定. 内皮型·一氧化氮合酶(eNOS) mRNA 和诱导型一氧化氮合酶(iNOS) mRNA 的表达用半定量逆转录聚合酶链式反应检测. **结果**：低氧引起内皮细胞的活力由正常条件下(93.0 ± 2.6) % 降至(85.5 ± 2.1) % ($P < 0.01$)，并导致内皮细胞 LDH 的释放由(41 ± 28) U/L 增加至(141 ± 68) U/L ($P < 0.01$). 丹酚酸 B 镁盐 5 mg/L 和 10 mg/L 能明显提高内皮细胞活力和降低 LDH 释放量. 同时，低氧还显著增加内皮细胞的胞内游离钙浓度和一氧化氮释放，并上调其 eNOS mRNA 和 iNOS mRNA 的表达 ($P < 0.01$). 丹酚酸 B 镁盐 5 mg/L 和 10 mg/L 能抑制其增加 ($P < 0.01$)，但在无钙的条件下，低氧对其均没有影响. **结论**：丹酚酸 B 镁盐能改善低氧引起的内皮细胞损伤，抑制低氧引起的内皮细胞内钙浓度的升高，一氧化氮释放，eNOS 和 iNOS mRNA 表达的增加. 丹酚酸 B 镁盐对一氧化氮产生和 eNOS mRNA 表达的抑制可能与其抑制低氧引起内皮细胞的胞外钙内流有关.

（责任编辑 朱倩蓉）