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Research Paper

Martentoxin, a large-conductance Ca^{2+}-activated K^+ channel inhibitor, attenuated TNF-α-induced nitric oxide release by human umbilical vein endothelial cells

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Abstract

Martentoxin, a 4,046 Da polypeptide toxin purified from the venom of the scorpion *Buthus martensii Karsch*, has been demonstrated to block large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels; however, its biological roles are still largely unknown. In the present study, we investigated the pharmacological effects of martentoxin on regulating the production of nitric oxide induced by TNF-α in human umbilical vein endothelial cells (HUVECs). We found that, 1, 10 and 100 μmol/L martentoxin decreased nitric oxide production by HUVECs exposed to 10 ng/mL TNF for 6, 12 and 24 hours. We further demonstrated that martentoxin inhibited the activity of iNOS and retarded the down-regulation of *eNOS* mRNA induced by TNF-α. Therefore, martentoxin could be a potential therapeutic agent for vascular diseases.

Keywords: martentoxin, *Buthus martensii Karsch*, nitric oxide, human umbilical vein endothelial cells (HUVECs)

INTRODUCTION

The Asian scorpion *Buthus martensii Karsch* (BMK), which is widely distributed from northwestern China to Mongolia and Korea, has been used in Chinese Traditional Medicine for thousands of years. The whole scorpions, scorpion tails and their extractions are effective in treating severe neurological disease such as epilepsy, apoplexy and facial paralysis apart from their use for soothing nerves and pains[1]. BMK venom is a rich source of substances, mainly neurotoxin proteins or peptides acting on various ionic channels in excitable cell membranes. Over the past decade, more than 70 different peptides, toxins or homologues have been isolated[2]. However, biological functions are still largely unknown.

Nitric oxide (NO), one of the most important vasoactive substances, is produced by nitric oxide synthase (NOS)[3] and can be released from the endothelium in certain vascular disease. Three members

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of NOS have been identified in mammals, including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), which have distinct functions and structural features[4]. Activities of nNOS and eNOS are regulated by Ca^{2+}-calmodulin and expressed constitutively in endothelial cells. iNOS is expressed in response to inflammatory and pro-inflammatory mediators, independent of intracellular Ca^{2+} levels[5]. In endothelial cells, alteration in membrane potential mediated by Ca^{2+}-activated K^+ channels is expected to affect Ca^{2+} influx and NO formation[6].

Previous studies suggested that BMK extracts (10 μg/mL) strongly inhibited TNF-α induced NO production and NOS expression, especially for iNOS[7], but which ingredient is involved in this process is unknown. Martentoxin, a 37-residue peptide purified from the BMK venom[8], belongs to subfamily 16 (α-KTx 16.2) toxin[9,10]. Martentoxin blocks BK_{Ca} and delays rectifying potassium channels in adrenal medulla chromaffin cells and hippocampal neurons, respectively[11]. The BK_{Ca} channels are present in mammals, insects and nematodes and participate in vascular regulation[12]. In humans, BK_{Ca} channels are present in the brain, bladder, pancreatic islets, cochlea and endothelial cells including human umbilical vein endothelial cells (HUVECs)[13]. Accumulating data indicated that Hyperpolarization as a result of the activation of BK_{Ca} channels is associated with NO release in endothelium[23]. Therefore, in the present study, we investigated the effects of BMK peptide martentoxin on NO production, iNOS activation and eNOS expression in HUVECS .

MATERIALS AND METHODS

Reagents

Scorpion venom collected by an electrical milking procedure was a lyophilized product purchased from Jiangsu Province, China. The crude venom powder was stored at -20°C until required. ÄKTA purifier 10 chromatography system, SOURCE 15 RPC 4.6/100 reversed-phase column and HiTrap SP FF (5 mL) ion-exchange columns were purchased from Amersham Biosciences (Uppsala, Sweden). C_8 reversed-phase column was bought from Kromasil (Bohus, Sweden). Trifluoroacetic acid (TFA), sodium acetate and acetonitrile of chromatographic grade were bought from Alfa Aesar (Ward Hill, USA) and Nanjing Chemical Reagent NO.1 Factory (Nanjing, China) and Merck (Schuchardt, Germany), respectively. Medium M199, trypsin, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone (Thermo scientific, USA). Recombinant human TNF-α was obtained from Peprothch (Rocky Hill, USA). Kit for the determination of total NO levels, iNOS were obtained from Nanjing Jiancheng Bioengineering Co. (Nanjing, China). FITC-conjugated goat anti-rabbit and 2-(4-amidinophenyl) -6-indolecarbamidine dihydrochloride (DAPI) were purchased from ZSGB-BIO (Beijing, China) and Sigma (St. Louis, MO, USA), respectively. ReverTra Ace qPCR RT Kit and THUNDERBIRD SYBR qPCR mix were bought from Toyobo (Osaka, Japan).

Ion-exchange and reverse phase chromatography

About 2 g BMK venom powder was dissolved in 40 mL 50 mmol/L sodium acetate (pH 4.9), and centrifuged at 12,000 *g* for 20 minutes. The supernatant was then loaded onto the Amicon Ultra-15 Centrifugal Filter Devices and centrifuged at 4,000 g for 30 minutes. The fractions were pooled for further purification. The pooled fraction collected in the previous step was applied on HiTrap SP FF ion-exchange column (5 mL) pre-equilibrated with 50 mmol/L CH3COONa buffer (PH 4.9). The samples were eluted with a 1-40% 1M NaCl gradient at a flow rate of 1 mL/min. The eluted fractions were monitored by UV280. For reverse-phase chromatography, the fraction from HiTrap SP FF column was loaded on C8 column (pre-equilibrated with 0.050% TFA) and the protein was then eluted with a 0-40% acetonitrile (containing 0.065% TFA) gradient at a flow rate of 1 mL/min, monitored by UV280.

Mass spectrometry

Determination of the molecular mass was carried out at the Research Centre for Proteome Analysis, Key Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China, by AutoFlex MALDI-TOF-TOF-MS (Bruker). For N-terminal sequencing, ABI Procise 491 Protein Sequencer at the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) was used to determine the N-terminal sequence of the purified protein.

Cell culture and identification

HUVECs were obtained from human umbilical cord veins (supplied by Nanjing Maternity and Child Health Care Hospital) by digestion with 0.1% collagenase type II (Sigma) as previously described[14]. The cells were cultured in M199 medium containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37°C in a fully humidified atmosphere containing 5% CO_2. The anti-von Willebrand factor antibody

Table 1 **The primer sequence used for real-time RT-PCR**

Gene name	Forward primer	Reverse primer	Accession Number
eNOS	CACATGTTTGTCTG CGG	GAGGGGCCTTCCAGATTAAG	NM_00603
GAPDH	GGGAAGCTCACTGGCATGGCCTTCC	CATGTGGGCCATGAGGTCCACCAC	NM_002046

was used to label blood vessels[15]. After one week of culture, the HUVECs were identified by immunofluorescence staining with anti-von Willebrand factor antibody[16,17]. HUVECs were rinsed three times with 0.1 M phosphate buffered saline (PBS) and then fixed in methanol after growing to 90% confluence. Cells were permeabilized in PBS containing 0.3% Triton X-100 (ZSGB-BIO, Beijing, China) for 30 minutes, rinsed in PBS and pre-incubated with 10% goat serum in PBS for 60 minutes at room temperature. The cells were then incubated in rabbit anti-FacVIII antibody overnight at 4°C. After several washes in PBS, secondary FITC-conjugated goat anti-rabbit antibodies were applied for 2 hours at room temperature in the dark. Nuclei were stained with fluorescent nucleic acid dye DAPI in the dark. After several washes, outgrowth cells were visualized using fluorescence microscope (Olympus IX70, Japan), adapted with Mercury lamp (Olympus).

Determination of NO production and iNOS activtiy

To evaluate the effect of martentoxin on NO production induced by TNF-α (10 ng/mL) in HUVECs, we treated cells with martentoxin (1, 10 and 100 μmol/L) and/or a non-specific NOS inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L). After 6, 12 and 24 hours of incubation, the HUVECs were collected for NO synthase activity assays or for protein extraction. Nitric oxide was detected according to Griess et al.[18], and nitrite concentrations were determined at 550 nM by using the standard solutions of sodium nitrite. The experiments were repeated at least three times. The NOS activity of HUVECs was measured as described previously[19]. Briefly, HUVECs were homogenized in ice-cold saline and centrifuged (3,000 g, 10 minutes). Supernatant was collected for testing tNOS and iNOS activities by using the NOS kits following manufacturer´s instructions. Additionally, HUVECs were treated with TNF-α (10 ng/mL), martentoxin (100 μmol/L), and martentoxin (100 μmol/L) + TNF-α (10 ng/mL), respectively. After 24 hours of incubation, the medium was collected for NO detection.

Real time RT-PCR

Total RNA was extracted from HUVECS by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 1 μg was reverse transcribed in a 20 μL reaction. The 20 μL of RT-reaction mixture and each primer set (***Table 1***) were prepared to detect *eNOS* mRNA expression by real time RT-PCR (ABI PRISM 7,300 sequence of detection system) with Thunderbird SYBR qPCR mix. The temperature profile was as follows: 94°C for 4 minutes, followed by 40 cycles of 94°C for 30 seconds, 59°C for 45 seconds and 72°C for 45 seconds. Each real-time RT-PCR reaction was performed in quadruplet. Fold changes in mRNA levels were calculated by using the $^{\triangle\triangle}$CT method and *GAPDH* was used as the reference gene. Specificity of PCR reaction was double-confirmed by electrophoresis and melting curve analysis.

Statistical analysis

Data are expressed as mean±S.D. for all experiments. Statistically significant differences between treatments and control were determined by one-way or two-way ANOVA and LSD multiple comparison procedure. All tests of statistical significance were two-sided and $^{*}P < 0.05$ was considered statistically significant.

RESULTS

Crude venom isolation and purification

As shown in ***Fig. 1A***, a total of 8 peaks were eluted from the initial HiTrap SP FF column separation of venom and peak 3 was collected. Upon reverse-phase

Table 2 **Amino acid sequence comparison of martentoxin from Buthus martensii Karch with other mammalian toxins**

Name	Sequence	Sequence Identity
Martentoxin	FGLIDVKCFASSECWTACKKVTGSGQGKCQNNQCRCY	100%
KTX1	FGLIDVKCFASSECWIACKKVTGSVQGKCQNNQCRCY	94%
TmTX	LIDVKCFASSECWTACKKVTGSGQGKCQNNQCRCY	77%
BmKTX2	QFTNVSCSASSQCWPVCKKLFGTYRGKCMNSKCRCYS	57%
BmTX1	QFTDVKCTGSKQCWPVCKQMFGKPNGKCMNGKCRCYS	54%

chromatography, pooled peak 3 fraction from the first step was further separated into 12 peaks, and we collected peak 4 as shown in ***Fig. 1B***. Peptide was applied on a C8 reversed-phase column and a single peak was eluted (***Fig. 1C***). Through 2 steps combined with HiTrap SP FF ion-exchange chromatography and Source 15 RPC reverse-phase chromatography, about 0.015 g martentoxin were purified to homogeneity from 2 g crude venom. The yield of martentoxin was about 0.75%, higher than the previous report[8]. The molecular mass of martentoxin estimated by AutoFlex MALDI -TOF -TOF -MS was 4,059 Da. No other peptide was found during automated Edman degradation process. The 37 amino acid of martentoxin was obtained and sent for homology searching by Blast. By comparison with other toxins purified from *Buthus martensii* Karch venom, the results showed 100% sequence identity with martentoxin (***Table 2***).

Martentoxin markedly decreased TNF-α-induced NO release

Cultured HUVECs exhibited cobblestone-like appearance and contact-inhibition between cells under phase-contrast microscopy. Immunocytochemistry with anti-von Willebrand factor (vWF)[20] showed that more than 95% of cells were positive vascular endothelial cells (***Fig. 2***). To examine the effects of martentoxin on NO production, we treated cells with TNF-α for 2 hours. ***Fig. 3*** showed that TNF-α significantly increased the production of NO at 6, 12 and 24 hours while martentoxin (1, 10 and 100 μmol/L) markedly inhibited NO production induced by TNF-α. We also explored whether martentoxin exerted its effect on NO production. The data indicated that 100 μmol/L martentoxin, treated for 24 hours, obviously increased NO production, but when co-incubated with 10 ng/mL TNF-α, it markedly decreased NO production compared with the TNF-α treated group (***Fig. 3D***).

Martentoxin reduced iNOS activity mediated by TNF-α

Then, we investigated how martentoxin affects TNF-α mediated NO production. It is well known that TNF-α enhances iNOS activity and thus increases NO production[21]. Therefore, iNOS activities should be determined. As shown in Fig. 4, activity of iNOS modulated by TNF-α was reduced after treatment with 1, 10, and 100 μmol/L martentoxin for 6, 12 and 24 hours.

Effects of martentoxin on TNF-α mediated *eNOS* gene expression

As eNOS is believed to mediate important vasoprotective effect[26], we investigated the effects of martentoxin on eNOS expression. Our results indicated

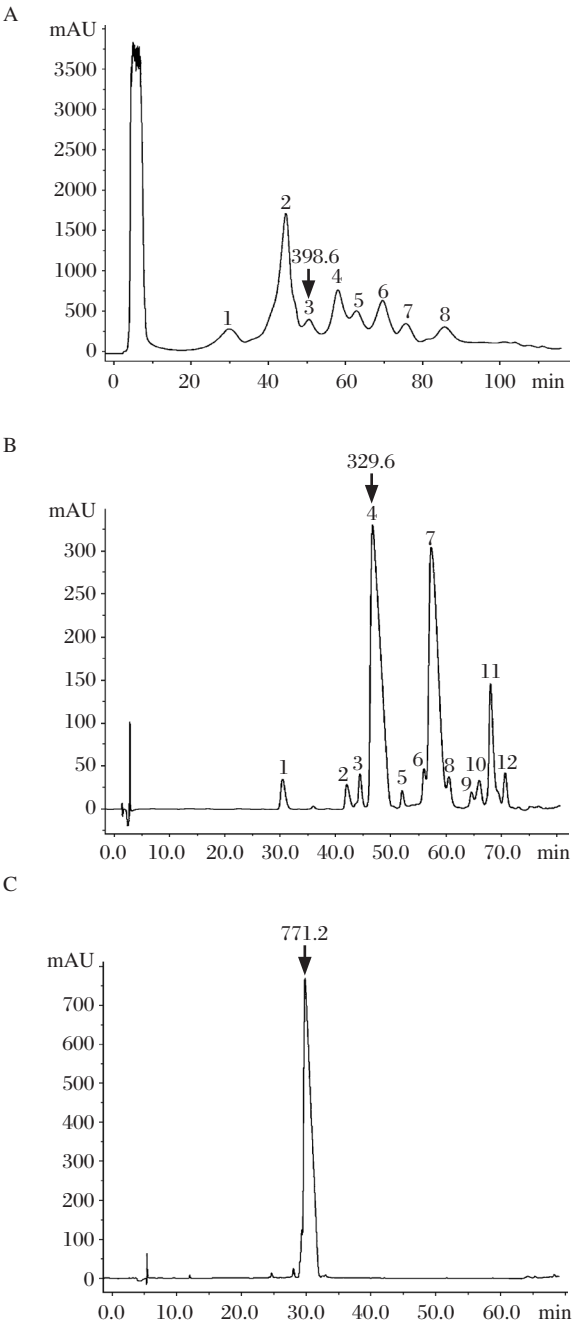

Fig. 1 **Isolation and purification of martentoxin from *Buthus martensii Karch*.** A: Cation exchange chromatography. The column (HiTrap SP FF) was equilibrated with 50 mmol/L CH_3COONa, pH 4.9 buffer, and eluted with a linear gradient of 1 M NaCl, pH 4.9. B: Reverse-phase chromatography. The fraction from (A) was lyophilized and dissolved in ddH_2O and purified on SOURCE 15 RPC column equilibrated with buffer A (0.65% TFA). Elution was done by applying a linear gradient of buffer B (0.05%TFA in acetonitrile). C: The fraction from (B) was lyophilized and dissolved in ddH_2O and loaded on the C_8 column. The protein was equilibrated with buffer A and eluted with buffer B.

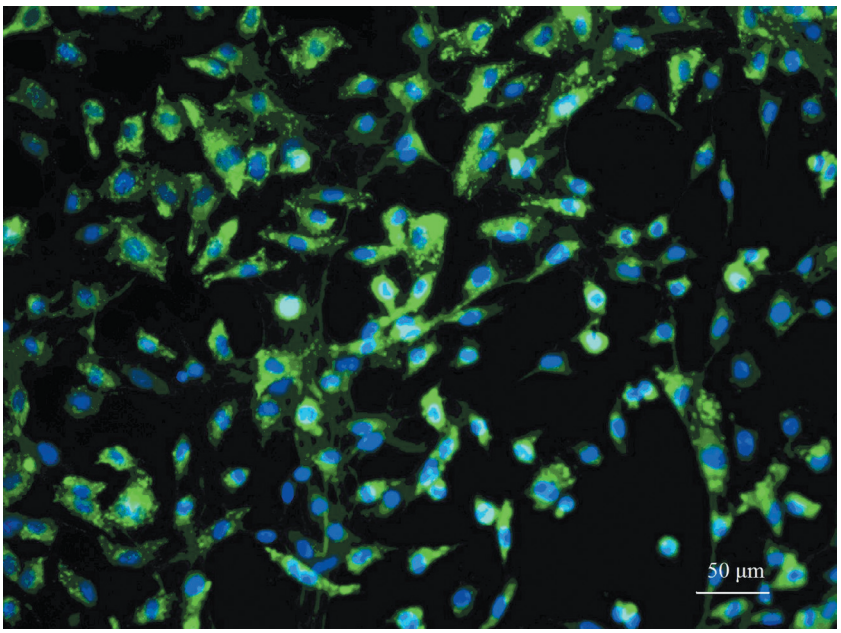

Fig. 2 **Photomicrograhps of 7-day-old cultures of human umbilical vein endothelial cells (HUVECs) immunostained with anti-von willebrand factor and anti-rabbit IgG-FITC (green).** Nuclei were stained with DAPI (blue). Scale bar, 50 μm.

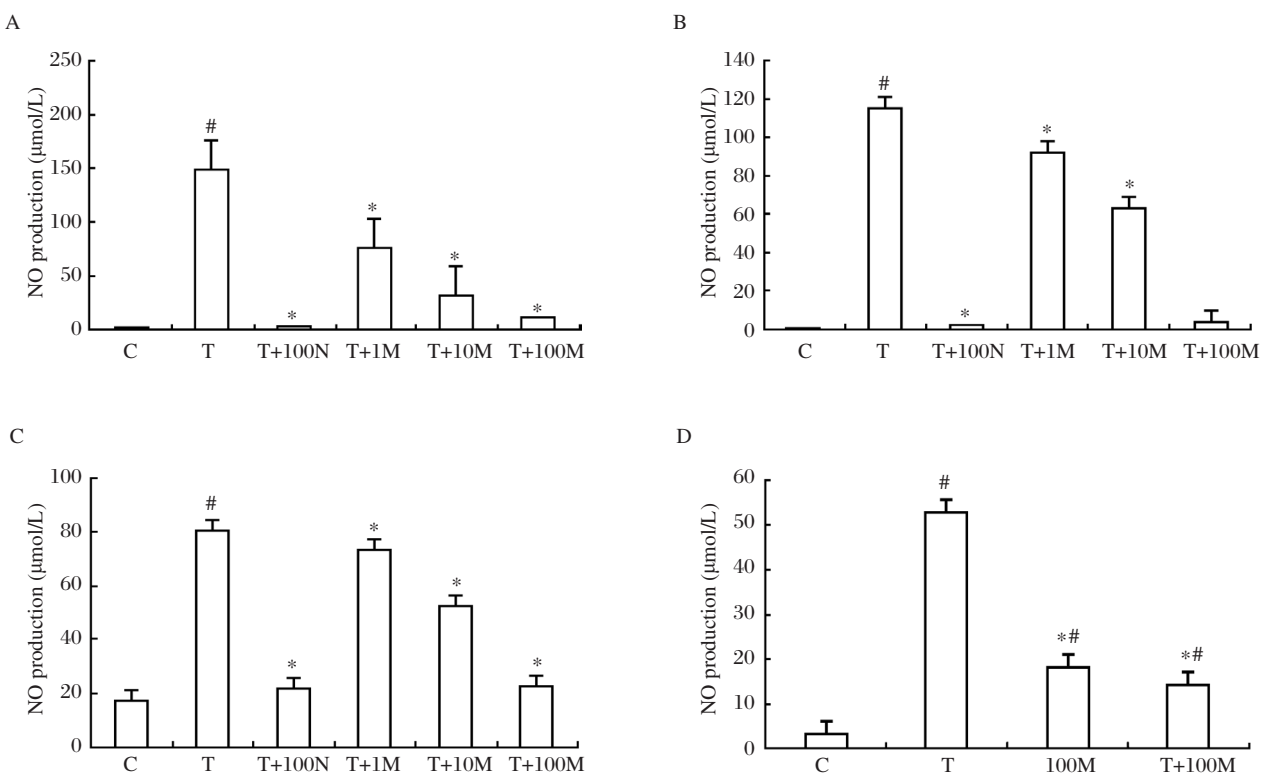

Fig. 3 **Inhibitory effect of martentoxin (1, 10, 100 μmol/L) and L-NAME (100 μmol/L) on NO production in HUVECs induced by TNF-α (10 ng/mL).** A: Martentoxin inhibited NO production induced by TNF-α after 6 hours. B: Martentoxin inhibited NO production induced by TNF-α after 12 hours. C: Martentoxin inhibited NO production induced by TNF-α after 24 hours. D: The effects of martentoxin on NO production. # and * indicate significant differences ($P < 0.05$) compared with the control and condition stimulated by TNF-α. Each value denotes the mean ± SD ($n = 5$). C: control; T: TNF-α (10 ng/mL); T+100N: TNF-α (10 ng/mL)+L-NAME (100 μmol/L); T+1M: TNF-α (10 ng/mL)+ Martentoxin (1 μmol/L); T+10M: TNF-α (10 ng/mL)+ Martentoxin (10 μmol/L) ; T+100M: TNF-α (10 ng/mL)+Martentoxin (100 μmol/L).

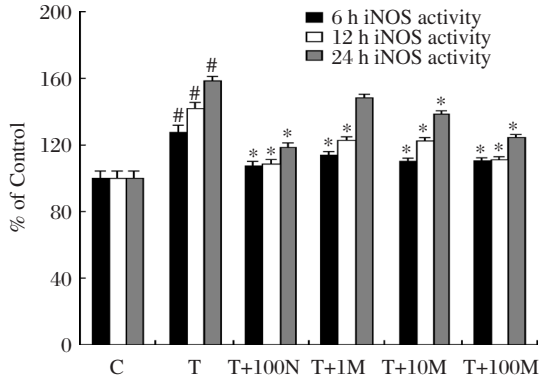

***Fig. 4* Martentoxin (1, 10, 100 μmol/L) down-regulated the activity of iNOS mediated by TNF-α after treatment for 6, 12 and 24 hours.** # and * indicate significant differences compared with the control and condition stimulated by TNF-α ($P < 0.05$); each data represents mean ± SD at least three separate experiments. C: control; T: TNF-α (10 ng/mL); T+100N: TNF-α (10 ng/mL)+L-NAME (100 μmol/L) ; T+1M: TNF-α (10 ng/mL)+ Martentoxin (1 μmol/L); T+10M: TNF-α (10 ng/mL)+ Martentoxin (10 μmol/L) ; T+100M: TNF-α (10 ng/mL)+Martentoxin (100 μmol/L).

that TNF-α (10 ng/mL) downregulated *eNOS* mRNA expression in HUVECs, while treatment with martentoxin (100 μM) or co-incubation with both martentoxin and TNF-α (10 ng/mL) showed no apparent changes in *eNOS* mRNA expression (***Fig. 5***).

DISCUSSION

Large-conductance Ca^{2+} activated K^+ (BK_{Ca}) channels play important roles in the regulation of membrane excitability. The importance of BK_{Ca} channels

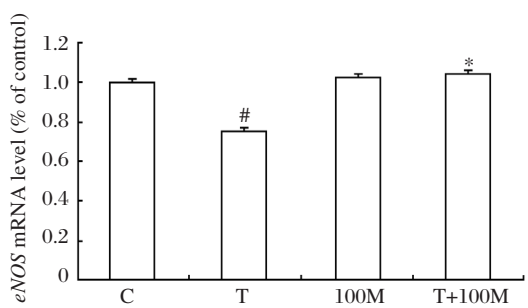

***Fig. 5* Martentoxin significantly retarded TNF-α (10 ng/ml) down-regulated *eNOS* mRNA expression.** Each data point represents mean±SD of mRNA levels from at least three separate experiments in which treatments were performed in triplicates. # and * indicate significant differences compared with the control and condition stimulated by TNF-α ($P < 0.05$), each data represents mean±SD at least three separate experiments. C: control; T: TNF-α (10 ng/mL); 100M: Martentoxin (100 μmol/L);T+100M: TNF-α (10 ng/mL) + Martentoxin (100 μmol/L).

in regulation of vascular tone, determination of action potential duration and frequency, and neurotransmitter release has been well documented[24]. Previous studies suggested that BKCa channels expressed in intact vascular endothelium are associated with NO release. In the present study, we isolated a 4.059 kD peptide from BMK venom,, a large-conductance Ca^{2+}-activated K^+ channels inhibitor- martentoxin. It is well known that TNF-α up-regulates the expression of arginase which is the mediator of nitric oxide through L-arginine-NO pathway in endothelial cells[22]. Our data suggested that martentoxin decreased the biosynthesis of total NO in HUVECs induced by TNF-α, which is considered to play a key role in inflammatory and immune response. To test the specific effects of martentoxin on TNF-α induced NO production, varied doses of martentoxin (1, 10 and 100 μmol/L) were used. The results showed that martentoxin inhibited TNF-α mediated NO production in a dose-depend manner not only in 6 hours, but also in 12 and 24 hours. In addition, a non-specific inhibitor of nitric oxide synthase, L-NAME, was used in the present experiment. As we expected, TNF-α stimulated NO production and L-NAME substantially reduced the release of NO induced by TNF-α in HUVECs.

NO is a modulator of vascular inflammation. Hama et al. indicated that proinflammatory cytokines and lipopolysaccharide (LPS) increased iNOS expression, which impaired vascular integrity in chronic inflammation[25]. Similar to Hama´s results, our data suggested that proinflammatory cytokine TNF-α, significantly promoted iNOS activities and facilitated NO release. Moreover, we foumd that TNF-α-induced iNOS activities and NO release were attenuated by martentoxin. It was worth mentioning that 100 μM martentoxin elicited a pronounced release of NO from the HUVECs. Xu reported[28] that endotoxemic BK β1-KO mice had higher plasma TNF-α and iNOS expression in the heart. Moreover, Tao[29] indicated that accessory β1 subunit exerted important effects on martentoxin-mediated BK channel (α+β1) activities implying that accessory β subunits may be targets for martentoxin in TNF-α-mediated NO activities. However, the mechanisms deserved further investigations.

Traditionally, eNOS is believed to be the primary source of NO in the endothelium and mediates important vasoprotective and immunomodulatory effects[26]. Our data showed that TNF-α substantially downregulated *eNOS* mRNA expression. This result confirmed and supported the conclusion by Zemse that TNF-α decreased eNOS expression in mouse endothelium [27]. In the present study, we found that martentoxin retarded the downregulation of eNOS

expression induced by TNF-α. Thereby, martentoxin could be a potential therapeutic agent for vascular diseases. Firstly, it significantly reduced TNF-α mediated NO production and attenuated iNOS activity, which has been demonstrated to be highly correlated with HUVECs inflammatory response. Secondly, it markedly retarded eNOS downregulation mediated by TNF-α. Different from eNOS, nNOS is much less abundant than eNOS and is predominantly in resting endothelial cells [26], so we did not detect nNOS expression in HUVECs.

In summary, we demonstrated that martentoxin, as a peptide purified from the venom of scorpion BKM, decreased NO production and retarded iNOS activation in HUVECs induced by TNF-α. Furthermore, martentoxin upregulated eNOS expression. However, the exact mechanisms of martentoxin induced endothelial response and the contribution of iNOS and eNOS to this response are still unknown. Further research will focus on the mechanisms of protective effects of martentoxin in inflammatory vascular diseases.

References

[1] Liu YF, Ma RL, Wang SL, Duan ZY, Zhang JH, Wu LJ, et al. Expression of an antitumor-analgesic peptide from the venom of Chinese scorpion Buthus martensii karsch in Escherichia coli. *Protein Expr Purif* 2003; 27: 253-8.

[2] Liang S. An overview of peptide toxins from the venom of the Chinese bird spider Selenocosmia huwena Wang [=Ornithoctonus huwena (Wang)], *Toxicon* 2004; 43: 575-85.

[3] Stuehr DJ. Mammalian nitric oxide synthases. *Biochim Biophys Acta* 1999; 1411: 217-30.

[4] Stuehr DJ, Santolini J, Wang ZQ, Wei CC, Adak S.Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem* 2004; 279: 36167-170.

[5] Stuehr DJ, Griffith OW. Mammalian nitric oxide synthases. *Adv Enzymol Relat Areas Mol Biol* 1992; 65: 287-346.

[6] Busse R, Lückhoff A, Mülsch A. Cellular mechanisms controlling EDRF/NO formation in endothelial cells. *Basic Res Cardiol* 1991; 86 (S2): 7-16.

[7] Jin UH, Kim KS, Park SY, Chung KH, Kim DS, Chang YC, et al. Effect of Buthus martensi Karsch on aromatase activity and cytokine-inducted NOS and NO production in osteoblasts and leukaemic cell line FLG 29.1. *Immunopharmacol Immunotoxicol* 2006; 28: 241-58.

[8] Cao ZY, Shen WQ, Pan YP, Xiao X, Liu XM, Wang XL, et al. Purification, characterization of two peptides from Buthus martensi Karch. *J Pept Res* 2003; 62: 252-9.

[9] Wang Y, Chen X, Zhang N, Wu G, Wu H. The solution structure of BmTx3B, a member of the scorpion toxin subfamily alpha-KTx 16. *Proteins* 2005; 58: 489-97.

[10] Wang YH, Cao ZY, He WY, Yan XZ, Liu X, Liu HY, et al. 1H-NMR signal assignments and secondary structure analysis of martentoxin. *J Asian Nat Prod Res* 2006; 8: 511-8.

[11] Shi J, He HQ, Zhao R, Duan YH, Chen J, Chen Y, et al. Inhibition of martentoxin on neuronal BK channel subtype (alpha+beta4): implications for a novel interaction model. *Biophys J* 2008; 94: 3706-13.

[12] Ledoux J, Werner ME, Brayden JE, Nelson MT.Calcium-activated potassium channels and the regulation of vascular tone. *Physiology (Bethesda)* 2006; 21: 69-78.

[13] Kestler HA, Janko S, Häussler U, Muche R, Hombach V, Höher M, et al. A remark on the high-conductance calcium-activated potassium channel in human endothelial cells. *Res Exp Med (Berl)* 1998; 198: 133-43.

[14] Jaffe EA, Grulich J, Weksler BB, Hampel G, Watanabe K. Correlation between thrombin-induced prostacyclin production and inositol trisphosphate and cytosolic free calcium levels in cultured human endothelial cells. *J Biol Chem* 1987; 262: 8557-65.

[15] Uchihara T, Kondo H, Akiyama H, Ikeda K. Single-laser three-color immunolabeling of a histological section by laser scanning microscopy: application to senile plaque-related structures in post-mortem human brain tissue. *J Histochem Cytochem* 1995; 43: 103-6.

[16] al-Mohanna F, Collison K, Parhar R, Kwaasi A, Meyer B, Saleh S, et al. Activation of naive xenogeneic but not allogeneic endothelial cells by human naive neutrophils: a potential occult barrier to xenotransplantation. *Am J Pathol* 1997; 151: 111-20.

[17] Urbán Z, Riazi S, Seidl TL, Katahira J, Smoot LB, Chitayat D, et al. Connection between elastin haploinsufficiency and increased cell proliferation in patients with supravalvular aortic stenosis and Williams-Beuren syndrome. *Am J Hum Genet* 2002; 71: 30-44.

[18] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. *Anal Biochem* 1982; 126: 131-8.

[19] Sheng L, Qian Z, Shi Y, Yang L, Xi L, Zhao B, et al. Crocetin improves the insulin resistance induced by high-fat diet in rats. *Br J Pharmacol* 2008; 154: 1016-24.

[20] Li QS, Liu Y, Feng ZJ, Lu ZS, Qian MZ. The molecular mechanism of apoptosis of human umbilical vein endothelial cells induced by monocyte chemotacitic protein-1. *Sheng Li Xue Bao* 2010; 62: 63-8.

[21] Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sci* 2004; 75: 639-53.

[22] Gao X, Xu X, Belmadani S, Park Y, Tang Z, Feldman AM, et al. TNF-alpha contributes to endothelial dysfunction by upregulating arginase in ischemia/reperfusion injury. *Arterioscler Thromb Vasc Biol* 2007; 27: 1269-75.

[23] Vang A, Mazer J, Casserly B, Choudhary G. Activation

of endothelial BKCa channels causes pulmonary vasodilation. *Vascul Pharmacol* 2010; 53: 122-9.

[24] Salkoff L, Butler A, Ferreira G, Santi C, Wei A. High-conductance potassium channels of the SLO family. *Nat Rev Neurosci* 2006; 7: 921-31.

[25] Hama S, Takeichi O, Fujisaki K, Tanabe N, Maeno M, Ochiai K. Nitric oxide attenuates vascular endothelial cadherin-mediated vascular integrity in human chronic inflammation. *Clin Exp Immunol* 2008; 154: 384-90.

[26] Chakrabarti S, Chan CK, Jiang Y, Davidge ST. Neuronal nitric oxide synthase regulates endothelial inflammation. *J Leukoc Biol* 2012; 91: 947-56.

[27] Zemse SM, Chiao CW, Hilgers RH, Webb RC. Interleukin-10 inhibits the in vivo and in vitro adverse effects of TNF-alpha on the endothelium of murine aorta. *Am J Physiol Heart Circ Physiol* 2010; 299: H1160-7.

[28] Xu H, Wang Y, Garver H, Galligan JJ, Fink GD.Vascular BK channel deficiency exacerbates organ damage and mortality in endotoxemic mice. *J Cardiovasc Pharmacol* 2012, 59: 207-14.

[29] Tao J, Shi J, Yan L, Chen Y, Duan YH, Ye P, et al. Enhancement effects of martentoxin on glioma BK channel and BK channel (α+β1) subtypes. *PLoS One* 2011, 6: e15896.