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

Jun Wang\(^{\Delta}\), Wenyi Qian\(^{\Delta}\), Qing Zhu, Jian Chen, Fei Huan, Rong Gao, Hang Xiao

Department of Toxicology, School of Public Health, Nanjing Medical University, Nanjing, Jiangsu 210029, China.

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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-\(\alpha\) in human umbilical vein endothelial cells (HUVEC). We found that, 1, 10 and 100 \(\mu\)mol/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-\(\alpha\). 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...
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\(^8\). 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\(^3\). 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 \(\mu g/mL\)) strongly inhibited TNF-\(\alpha\) induced NO production and NOS expression, especially for iNOS\(^7\), but which ingredient is involved in this process is unknown. Martenotoxin, a 37-residue peptide purified from the BMK venom\(^8\), belongs to subfamily 16 (\(\alpha\)-KTx 16.2) toxin\(^9,10\). Martenotoxin 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 martenotoxin 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. AKTA 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-\(\alpha\) 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-aminophenyl) -6-indoledcarbamidine 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 \(\mu g/mL\) streptomycin at 37°C in a fully humidified atmosphere containing 5% \(CO_2\). The anti-von Willebrand factor antibody
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 activity**

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 NO 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 △△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 1 The primer sequence used for real-time RT-PCR**

| Gene name | Forward primer | Reverse primer | Accession Number |
|-----------|----------------|----------------|------------------|
| eNOS      | CACATGTGGTGTCGCG | GAGGGGCCCTCAGATTAG | NM_006005       |
| GAPDH     | GGGAAGCTACTGCATGGCCCTCC | CATGTTGGCCATGAGTCCACCAC | NM_002046       |

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

| Name    | Sequence             | Sequence Identity |
|---------|----------------------|-------------------|
| Martentoxin | FGLIDVKCFASSECWTACKKVTGSQGKCMNGKRCY | 100%              |
| KTX1 | FGLIDVKCFASSECW1ACKKVTGSQGKCMNGKRCY | 94%              |
| TmTX | LIDVKCFASSECWTACKKVTGSQGKCMNGKRCY | 77%              |
| BmKTX2 | QFTNVSCASQCPVCKMFYRGGCMNGKRCYS | 57%              |
| BmTX1 | QFTDVKCGSKQCWPVCKQFMNGKCMNGKRCYS | 54%              |
Effects of martentoxin on NOS and NO production

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 Hi-Trap 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 vasopro-
**Fig. 2** Photomicrographs 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).
Effects of martentoxin on NOS and NO production

**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 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 found 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$^{[26]}$ 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.

**Fig. 4** Martentoxin (1, 10, 100 μmol/L) downregulated 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).

**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).
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.

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