Vasodilation of BCT is Associated with Inhibition of PKCα Translocation and LC20 Phosphorylation

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We have previously reported that the vasodilatory effect of BanhabackchulChunma-tang(半夏白朮天麻湯; BCT), a herbal formula, and its mechanism might be associated with at least in part NO pathway. In the present study, we studied the influence of BanhabackchulChenuma-tang (BCT) on the phosphorylation of LC20, in parallel, the distribution of α-protein kinase C(PKCα) by phenylephrine was monitored using laser scan confocal immunofluorescent microscopy in freshly isolated ferret portal vein smooth muscle living single cells. Phenylephrine stimulation induced LC20 phosphorylation and translocation of PKCα. However, BCT dephosphorylated LC20 phosphorylation and inhibited the translocation of PKCα. Our results demonstrate that the mechanism of relaxant effect of BanhabackchulChunma-tang inhibition is associated with inhibition of PKCα activation and LC20 phosphorylation.

Key words : BanhabackchulChunma-tang(半夏白朮天麻湯), ferret portal vein, PKCα, LC20, vasodilation

Introduction

Although it is generally recognized that the primary mechanism of smooth muscle contraction involves phosphorylation of LC20, PKC activation is also believed to affect the contractile state of smooth muscle[1]. The predominant isoform of PKC in ferret portal vein smooth muscle cells is PKCα[2] and in response to activation of cell surface receptors, PKCα is translocated from cytosol to plasma membrane in vascular smooth muscle cells[1]. Besides, LC20 is also phosphorylated by protein kinase C at serine-1 and 2 and threonine-9 which is associated with inhibition of ATPase[3].

BanhabackchulChunma-tang(BCT), which is composed of 14 herbal drugs, has long been used as a formula in oriental countries such as China, Japan and Korea for various purpose. It has been known to exert effects against headache, dizziness, vertigo, and hypertension due to circulation disorders[4,5]. We have previously studied the involvement of NO pathway in vascular relaxation effect of BCT[6]. However, a detailed study of the signal transduction of its efficacy has not been conducted. The aim of this study was to investigate whether inhibition of PKCα translocation and dephosphorylation of LC20 are associated with the possible mechanism of the relaxant effect of BCT.

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The aim of this study was to investigate whether vasodilation effect of BCT on vasocontraction is associated with inhibition of PKCα translocation and LC20 phosphorylation.

Materials and Methods

1. Herbal Composition and Preparation of Extract

BanhabackchulChunma-tang(BCT) is a mixture of herbal drugs, which was obtained from the Oriental Herbal Center(OHC) of the Orientl Medical Hospital of Dongguk Univ. College of Orientl Medicne(Kyoungju, Korea). The composition of the mixture is as follows: Pinelliae Rhizoma (5.6g), Citri Pericarpium(5.6g), Hordei Fructus Germinatus (5.6g), Atractylodis Macrocephalae Rhizoma(3.75g), Massa Medicata Fermentata(1.875g), Gastrodiae Rhizoma(1.875g), Alismatis Rhizoma(1.875g), Atractylodis Rhizoma(1.875g), Ginseng Radix(3.75g), Astragali Radix(3.75g), Poria(3.75g), Zingiberis Siccatum Rhizoma(3.75g), Phellodendri Cortex (3.75g), Uncariae Ramulus et Uncus(3.75g). For water extraction of BCT, BCT(260g) was mixed with 1,200ml of distilled water and extracted under reflux for 2hrs by boiling the formula. The extract was filtered with a Whatman filter paper. The filtrate was concentrated to about 100 ㎖ with a rotary evaporator at 50℃ under vacuum and freeze-dried to dryness.

2. Tissue Preparation

All procedures were performed according to protocols approved by the Institutional Animal Care and Use

Committee. Ferrets were killed by an overdose of chloroform, and the portal vein was quickly removed to a dissection dish filled with oxygenated physiological saline solution (PSS) at 22 ℃. The tissue was cleaned of connective tissue and opened longitudinally. The tissue was then cut into 3mm-wide strips and attached to a force transducer for contractility experiments as previously described[7] or used for single cell isolation as described in the following section.

3. Tension Measurement

The strips were placed in organ chamber with oxygenated(95% O^2-5%CO_2) PSS at 22℃. After 1hr equilibration in oxygenated PSS, vessels were stimulated with 10-5M phenylephrine. When the vessel reached a steady-state contraction, and then various concentration(0.025mg/ml, 0.125mg/ml and 0.25mg/ml) of water extract of BCT were added. The vessel tones were recorded in response to different concentration of BCT in the presence or absence of endothelium.

4. Preparation of Single Cells

Single cells from ferret portal vein were enzymatically isolated using a modification of a previously published method8). The portal vein was cut into small pieces (2 ㎜ x 2 ㎜) and placed in a siliconized flask containing digestion medium. For each 50 mg of portal vein (wet weight), the digestion medium A consisted of 4.2 mg CLS 2 collagenase (type II, 228 U/mg), 5.6 mg elastase (Grade II, 3.65 U/mg) and 5,000 U soybean trypsine inhibitor(type II-S) in 7.5 ml of Ca^{2+}-Mg^{2+}-free Hanks' balanced salt solution(HBSS). The tissue pieces were incubated in a shaking water bath at 34℃ under an atmosphere of pure oxygen for 40 min. The pieces ewre then filtered on a nylon mesh, rinsed with 10ml of cold 0.2% BSA containing Ca^{2+}-Mg^{2+}-free HBSS, and reincubated for 20 min in digestion medium B, i.e. the same digestion solution except for a decreased in the amount of collagenase to 2mg. After filtering and rinsing with 10ml of cold 0.2% BSA containing Ca^{2+}-Mg^{2+}-free HBSS, the dissociated cells were poured over glass cover slips, and plated for 40 min on the ice. For all experiments, isolated cells were first tested to confirm that they shortened in response to phenylephrine.

5. PKCα Translocation(Digital imaging)

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and reacted with mouse monoclonal anti-αPKC antibody(1:500 Transduction Laboratories) followed by a goat anti-rabbit Rhodamine Red-X secondary antibody (1:500 Molecular Probes) and mounted with Fluorsave (Calbiochem, San Diego, CA) before analysis. Images were obtained using a Kr/Ar laser (Radiance 2000) scanning confocal microscope equipped with Nikon X-60 (NA1.4)/ 40X (NA 1.4) oil immersion objectives. Images were recorded with Laser sharp 2000 for Windows NT. A previously described ratio analysis was performed to determine the relative distribution of PKCα within each cell and to normalize for possible differences in staining efficiency between cells

6. Measurements of LC20 Phosphorylation

Muscle strips were quick-frozen by immersion in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol(DTT). Tissues were brought to room temperature in acetone/TCA/DTT, then ground with glass pestles, and washed 3 times with ether to remove TCA. Tissues were extracted in a urea sample buffer as previously described and run on 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and subjected to immunoblot with a specific LC20 antibody (1:1500, Sigma). Anti-mouse IgG (Goat) conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Calbiochem). Bands were detected with enhanced chemiluminescence(ECL)(Supersignal, Pierce) visualized on films and then phosphorylated and unphosphorylated LC20 were analyzed by NIH Images.

7. Solutions and Materials

PSS contained (in mM): 120 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4 and 11.5 dextrose at pH 7.4 when bubbled with 95% O_2 + 5% CO_2. HBSS contained (in mM): 137 NaCl, 5.4 KCl, 0.44 $KH2PO_4$, 0.42 NaH_2PO_4, 4.17 $NaHCO_3$, 5.55 glucose, 10 HEPES, pH 7.4. PBS-Tween solution contained (in mM): 80 Na_2HPO_4, 20 NaH_2PO_4, 100 NaCl and 0.05% Tween. Phenylephrine (PE) were purchased from Sigma. General laboratory reagents were of analytical grade or better were purchased from Sigma and Fisher Scientific.

8. Statistics

All values given in the text are mean±SE. Differences between means were evaluated using a Student's test. Significant differences were taken at the $P<0.05$ level.

Results

1. Dose dependent relaxation effect of BCT

We investigated the effects of BCT on phenylephrine-induced contraction using both in the presence and absence of endothelium in different concentration of dose. BCT decreased

the contraction in dose-dependent manner. The extent of relaxation in responce to BCT in both with and without endothelium were 20.13±2.32 and 7.92±3.41 at 0.025mg/ml, 36.94±3.89 and 11.81±3.41 at 0.125mg/ml, 51.95±3.40 and 32.97±5.14 at 0.25mg/ml, respectively. The magnitude of vasodilation of BCT in the presence of endothelium was significantly($p<0.05$) higher than that in the absence of endothelium.

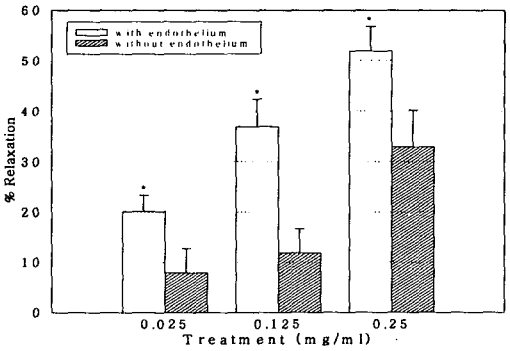

Fig. 1. Effects of BCT in the cumulative concentration on phenylephrine-induced contraction of ferret portal vein. The tension was measured in the presence and absence of endothelium. Each data point is a mean of results from 4 to 7 separate experiments. *p<0.05

2. Effect of BCT on LC20 phosphorylation

The basal level of LC20 phosphorylation in ferret portal vein at 22℃ was 0.22±0.010 mol Pi/mol LC20(n=4). LC20 phosphorylation level reached 0.25±0.021 mol Pi/mol LC20 after 35 min activation by phenylephrine. However, 0.25㎎/㎖ BCT treatment for 35 min in the cumulative concentration decreased significantly($p<0.05$) LC20 phosphorylation to 0.14±0.011 mol Pi/mol LC20. This level was under basal level(Fig. 2).

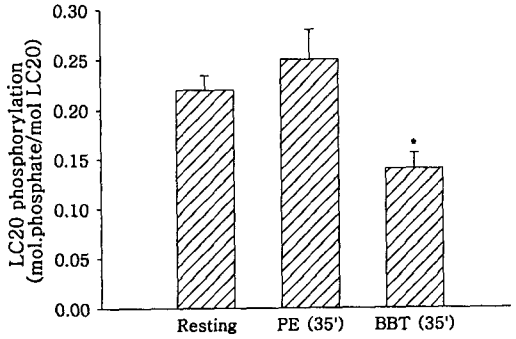

Fig. 2. Effect of BCT on phenylephrine-induced LC20 phosphorylation in ferret portal vein. All data are steady-state measurements. Each data point is a mean of results from 4 to 6 separate experiments. *P<0.05.

3. Effect of BCT on Localization of α-protein kinase C(PKCα)

As shown in Fig. 3, PKCα translocates from the cytosol to the vicinity of the cell membrane upon addition of phenylephrine. This translocation was inhibited when 0.1mg/ml of BCT was added. We quantitated the distribution of PKCα by measuring the ratio of confocal fluorescence at the surface of the cell to that at the cytosol of the cell. Line scans across the diameter of the cell were obtained from center optical sections of each cell. The nuclear area was excluded from analysis, and the numbers reported for each cell are an average from 3 line scans. Cells(7 to 10) were analyzed for each experimental group. The surface to cytosol fluorescence ratio significantly($p<0.01$) increased from 1.39±0.122 in resting cells to 2.36±0.125 at 5 min(figure not shown) and 2.38±0.348 at 8 min in stimulated cells with phenylephrine. When surface to cytosol ratio were calculated in BCT treated portal vein cells, it showed a significant($p<0.01$) decrease in quantification to 1.615±0.142 at 5min and 1.528±0.126 at 8min, respectively.

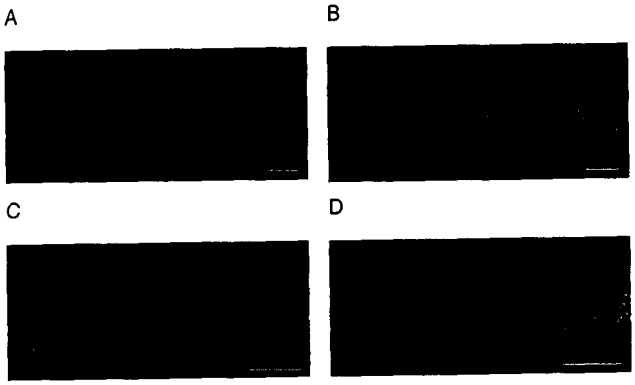

Fig. 3. Confocal images of α-protein kinase C(PKCα) in freshly isolated single living cells. Cells were fixed at rest(A) or stimulated with phenylephrine(10^{-5}M) for 8 min(B) or treated with phenylephrine containing BBT(0.1mg/ml) for 5 min(C) and 8min(D) before fixation. Bar = 10 ㎛.

Discussion

The main purpose of the study was to investigate whether PKCα translocation and LC20 phosphorylation are associated with the possible mechanism of the vsodilatory effect of BanhabackchulChunma-tang(BCT). BCT is a traditional herbal medicine used clinically in the Orient as a remedy for headache due to disorder of circulation and hypertension. The relaxation effects of BCT on phenylephrine-induced contraction of portal vein were measured in various dose. As shown in Fig. 1, BCT decreased the extent of phenylephrine-induced contraction in dose-dependent manner in both tissue types. But its relaxation effects much more effective in the presence of endothelium. This results are consistent with our previous report[6], suggesting the relaxation effect of BCT might be associated with NO pathway, but we can not exclude the possibility that BCT may cause vasorelaxation by blocking other signal transduction after receptor activation.

Phosphorylation of the 20 kDa myosin light chains(LC20) is generally accepted to be an important mechanism in regulating contractile activity of smooth muscle. Relaxation,

then, is generally the result of dephosphorylation of LC20 by myosin phosphatase[9,10]. It was reported that LC20 phosphorylation levels, in ferret aortic strips, reached peak at 30 s and then decreased gradually by 30 min but still increased significantly during phenylephrine stimulation[11]. In the present study, the treatment of BCT reduced the phosphorylation of LC20 by phenylephrine which was even below than that of basal level. There was also relationship between force and LC20 phosphorylation, decrease of LC20 phosphorylation caused about 52% relaxation on phenylephrine-induced contraction at the same 35 min time point. Thus such decreased phosphorylation of LC20 in the presence of BCT might decrease the sensitivity of MLCK for Ca^{2+}/calmodulin and result in relaxation.

Signaling pathways mediated by the protein kinase C(PKC) family of serine-threonine kinases are involved in the regulation of a wide variety of fundamental cellular processes, including smooth muscle contraction[12]. Recently, PKC has been identified as a family of at least 10 different isoforms with differing dependency on Ca^{2+}, phospholipids, and diacylglycerol for activation. The conventional PKC(cPKC) isoforms include α, βI, βII, and γ isoforms and require Ca^{2+} for activation, whereas the new PKC isoforms(nPKC) δ, ε, η, and θ, as well as the atypical PKC isoforms(aPKC), ζ and λ, do not require a significant increase in Ca^{2+} for their activation[12].

PKC activation plays an important role in the maintenance of phenylephrine-induced contraction in the ferret portal vein cells[1,2,13] and activation of PKC isozymes are generally associated with translocation[14]. It is demonstrated that the magnitude of translocation to the cell surface reached a maximal fluorescence ratio by 8 min[15]. In the present imaging study, as has been reported previously[15], PKCα showed a homogeneous distribution in the cytosol with a central area corresponding to the nucleus consistently unstained at rest and translocation from the cytosol to the vicinity of the surface membrane upon addition of phenylephrine. This translocation was inhibited by treatment of BCT.

Conclusion

The major findings of the present study were that BCT inhibited phenylephrine-induced LC20 phosphorylation and PKCα translocation. Accordingly, the relaxation effect of BCT is correlated with blockage of these signal transduction.

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