Effects of Butanol Fraction of Crataegi Fructus on the Translocation of PKCα and Myosin Phosphatase Subnits in Vascular Smooth Muscle

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LC20 phosphorylation and PKCα play an important role in modulation of contractile activity of smooth muscle. Besides, myosin phosphatase is also related with smooth muscle contraction in signaling pathways. We previously demonstrated that Crataegi Fructus inhibited phenylephrine-induced contraction and which might be implicated in nitrite formation(Son et al., 2002). In this study, we investigated the effects of butanol fraction of Crataegi Fructus(BFFC) on the localization of α-protein kinase C(PKCα) and myosin phosphatase subnits(MPs) in freshly isolated single ferret potal vein cells, and phosphorylation of LC20 during phenylephrine stimulation. In PKCα and MPs localization, BFFC blocked its translocation from the cytosol to the cell membrane by treatment of phenylephrine. BFFC have also dephosphorylated LC20 phosphorylation by phenylephrine stimulation under basal level, but no significant. These results indicate that the relaxation effect of BFFC is associated with inhibition of PKCα activation and MPs dissociation, and thus myosin phosphatase activity may be increased.

Key words : Smooth muscle contraction, Butanol fraction of Crataegi Fructus(BFFC), PKCα, MPs localization, LC20 phosphorylation

Introduction

Phosphorylation of the 20 kDa myosin light chains(LC20) is accepted as a general mechanism in regulation of contractile activity of smooth muscle[1]. The level of myosin phosphorylation reflects the activities of myosin light chain kinase and the myosin phosphatase(MP). MP in smooth muscle is composed of 3 subnits: a 38-kDa δ isoform of type 1 protein phosphatase catalytic subnit(PP1cδ), a large subnit of 110-130 kDa(MYPT, M130, targeting subnit), and a small subnit of 20-kDa(M20)[2,3]. At first, MP was thought to be constitutively active and not regulated. However, it has been demonstrated that MP can be regulated and is related with smooth muscle contraction[3,4]. Resently, it was demonstrated that the catalytic and targeting subnit of the phosphatase are dissociated from each other in an agonist-specific manner and this dissociation may provide a mechanism for the decreased phosphatase activity of phosphorylated myosin phosphatase in smooth muscle contraction[5]. In addition, activation of PKC plays an important role in the maintenance of phenylephrine- induced contraction in ferret portal vein[6,7]. PKC has been identified as a family of at least 10 different isoforms with differing dependency on Ca^{2+}, phospholipids, and diacylglycerol for activation[8]. The predominant isoform in ferret portal vein smooth muscle cells is PKCα[9] and in response to activation of cell surface receptors, PKCα is translocated from cytosol to plasma membrane in vascular smooth muscle cells[7]. On the other hand, study of medicinal herbs on smooth muscle contraction may provide the therapeutic applications for cardiovascular diseases in complementary medicine. Crataegi Fructus has long been used as a medicinal plant in oriental countries. It has been known to exert digestive and anti- hypertensive effects. We previously investigated that Crataegi Fructus inhibited phenylephrine-induced contraction and which might be implicated in nitrite formation[10]. However, the mechanisms underlying its efficacy in signal transduction are unknown. Thus, we examined the effects of butanol fraction of Crataegi Fructus(BFCF) on the localization of PKCα and myosin phophatase subnits(MPs) in isolated, permeabilized smooth muscle cells, and LC20 phosphorylation during phenylephrine stimulation.

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Materials and Method

1. Preparation of the Extract

Crataegi Fructus(Crataegus pinnatifida Bunge) is a herbal

drug, which was obtained from the Oriental Herbal Center (OHC) of the Orientl Medical Hospital of Dongguk University, College of Orientl Medicne (Kyoungju, Korea). For butanol fraction of Crataegi Fructus (CF), CF(200g) was extracted with 80% methanol for 4 hrs at 80℃. The extract was evaporated to eliminate methanol and added water and then sequentially extracted using a range of non-polar to polar solvents[11]. The butanol fraction was taken for further steps of purification(Fig. 1). As this butanol fraction of CF are water-insoluble, they are dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.1%.

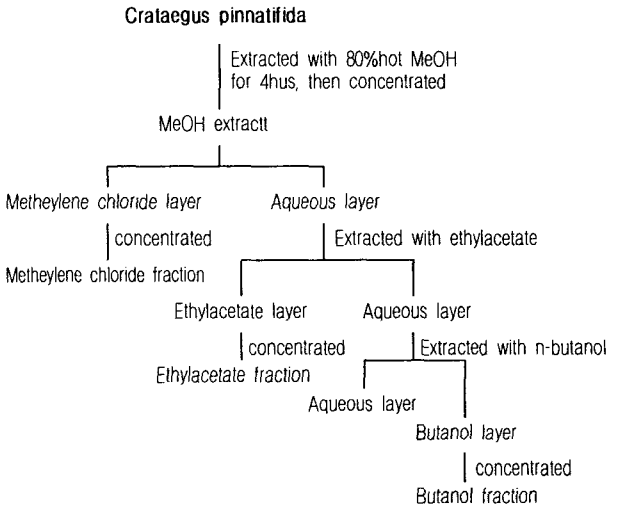

Fig. 1. Procedure of extraction and fractionation of n-butanol soluble fraction of Crataegus pinnatifida.

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). The tissue was then cut into strips and attached to a force transducer for contractility experiments as previously described[12] or used for singel cell isolation as described in the following section.

3. Preparation of single cells

Single cells from ferret portal vein were enzymatically isolated using a modification of a previously published method[13]. 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 trypsin 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 20mmin in digestion medium B, i.e. the same digestion solution except for a decrreased 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 coverslips, 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.

4. Digital imaging

Cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X-100, blocked with 10% goat serum and reacted with the appropriate primary antibody, i.e., PKC α (1:500 Transduction Laboratories), M130a(F38.130 1:10000 polyclonal[16] obtained from Covance, Richmond, Calif) and C-2PP1 δ (1:1500), respectively, followed by a goat anti-rabbit Rhodamine Red-X secondary antibody (1:500 Molecular Probes) and mounted with Fluorosave (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[14] was performed to determine the relative distribution of MYPT1 and PP1c subunits within each cell and to normalize for possible differences in staining efficiency between cells.

5. 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(Kim et al, 2000) 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.

6. Solutions and Materials

PSS contained (in mM): 120 NaCl, 5.9 KCl, 2.5 CaCl2, 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 KH_2PO_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.

7. 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. Then values given represent numbers of cells used in each experiment.

Results

1. Vasodilatory effect of BFCF

At the plateau of phenylephrine(10^{-5}M)-induced contraction, various cumulative concentrations of butanol fraction of BFCF were added. In the presence of endothelium, the relaxation amplitudes by BFCF were 18.81±2.52% (0.025mg/ml), 35.45±3.11% (0.125mg/ml) and 51.72±2.38% (0.25mg/ml), respectively. In contrast, the relaxation effect of BFCF were much less in the absence of endothelium, ie, 10.76± 2.71%, 24.11±2.46% and 32.54±1.95%, respectively(Fig. 1).

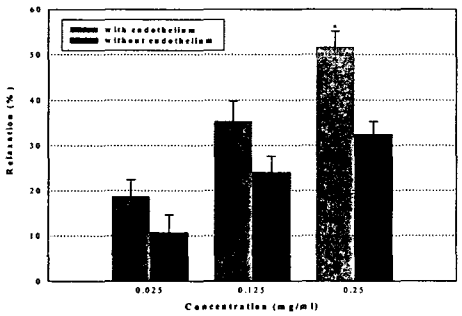

Fig. 1. Dose dependent relaxation effect of BFCF on phenylephrine-induced contraction in ferret portal vein in the presence and absence of endothelium. Values are mean ± S.D(n=5). *p<0.05.

2. BFCF dephosphorylates LC20 phosphorylation

Resting levels of LC20 phosphorylation in ferret portal vein was 0.22±0.01 mol Pi/mol LC20(n=4). LC20 phosphorylation level was increased slightly to 0.25±0.021 mol Pi/mol LC20 after 35 min stimulation by phenylephrine(PE) at 22℃. When 0.25mg/ml of BFCF was treated as cumulative concentration, the phosphorylation levels reduced to 0.15± 0.012 mol Pi/mol LC20, but no significant.

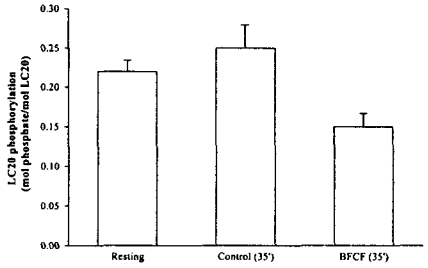

Fig. 2. Effect of BFCF on LC20 phosphorylation in ferret portal vein after PE stimulation. All LC20 phosphorylation levels were measured at plateau phase. Bar, 10 μm.

3. BFCF inhibits PKC α translocation

It was investigated the effect of BFCF on subcellular distribution of PKC α during activation by phenylephrine. Phenylephrine induced PKC α translocation from the sytosol to the cell membrane, but this translocation was inhibited by BFCF at all doses treated(Fig. 3A). We quantitated the distribution of PKC α by measuring the surface to cytosol confocal fluorescence ratio. Surface to cytosol fluorescence ratio were 1.51±0.072 at resting and 2.38±0.349 in phenlephrine stimulation. However, addition of BFCF decreased the ratio significantly to 1.55±0.052 at 0.05㎎/㎖, 1.49±0.039 at 0.1㎎/㎖ and 1.39±0.047 at 1.0㎎/㎖, respectively(Fig. 3B).

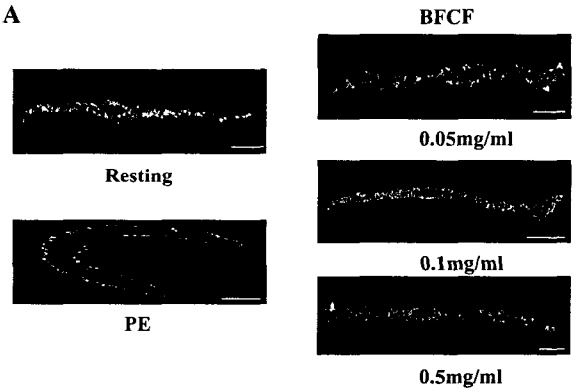

Fig. 3. A, Confocal images of PKC α in single ferret portal vein cells at rest or at the indicated concentration of BFCF with PE. Cells were treated with BFCF for 8 min, fixed, and then labled with anti α -PKC primary antibody, followed by a goat anti-rabbit Rhodamine Red-X secondary antibody. Bar, 10μm.

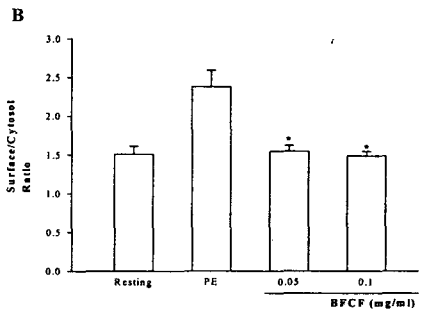

Fig. 3. B, Effect of BFCF on changes in surface to cytosol fluorescence ratio. Cells were treated with BFCF at indicated concentration. Values are mean ± S.D(n=7)* p<0.05.

4. BFCF prevents membrane targeting of PP1c

An example of the distribution of PP1c in single smooth muscle cells at rest is illustrated in fig. 4. Through the cell, the distribution of PP1c is homogeneous except nuclear. In the resting cells, the ratio for PP1c was 1.47 ± 0.034(n=7), and in the presence of phenylephrine, the ratio increased significantly ($p<0.01$) to 2.06 ± 0.079(n=5) at 5min, and then redistributed throughout the cells by 8 min. However, PP1c distribution remains fairly homogeneous by treatment of BFCF at all time points indicated. In the presence of BFCF, the surface to cytosolic ratio were 1.79 ± 0.11(n=5) at 5min and 1.62 ± 0.113(n=5) at 8min, respectively.

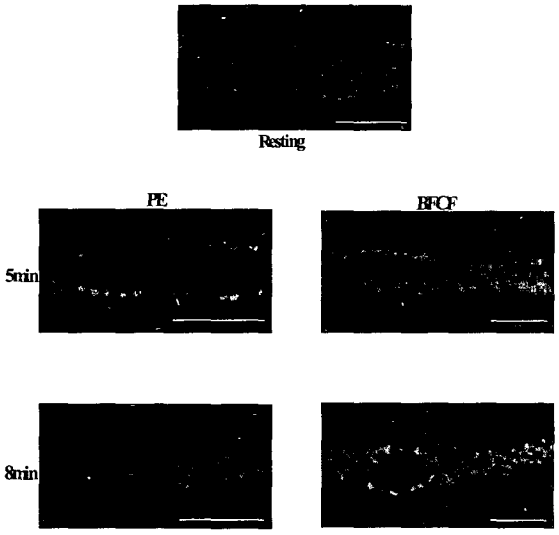

Fig. 4. Effect of BFCF on localization of PP1c in ferret portal vein single smooth muscle cells with PE. Bar, 10㎛.

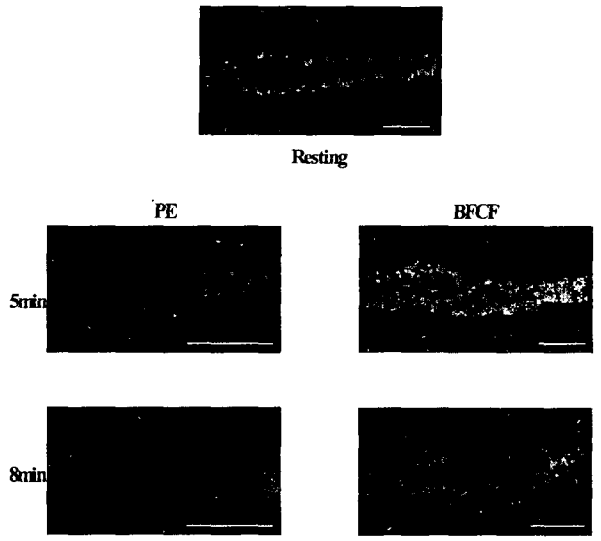

Fig. 5. Effect of BFCF on localization of MYPT1 in ferret portal vein single smooth muscle cells with PE. Bar, 10㎛.

5. BFCF prevents membrane targeting of MYPT1

In contrast, staining for MYPT1 was distributed throughout the cells at all time points investigated in the presence of phenylephrine(Fig. 5). The localization of MYPT1 was also seen homogeneous pattern with treatment of BFCF. The averaged surface to cytosolic distributions of MYPT1 were 1.38 ± 0.05 at rest(n=7), 1.52 ± 0.069(n=5) following phenylephrine stimulation for 8 min, and 1.49 ± 0.084(n=5) after BFCF treatment for 8 min.

Discussion

Crataegi Fructus, medicinal plant has been known to have anti-hypertensive effects. However, this putative efficacy and its mechanism are still unknown. So, we examined the effects of butanol fraction of Crataegi Fructus(BFCF), which exerted strong vasodilation effect in preliminary experiments, on distribution of α-protein kinase C(PKC α) and myosin phosphatase subnits(MPs) in freshly isolated ferret potal vein single smooth muscle cells, and phosphorylation of the 20 kDa myosin light chains(LC20) were investigated in phenylephrine induced contraction. Phosphorylation of LC20 plays an important role in regulation of contractile activity of smooth muscle[1]. Relaxation, then, is generally the result of dephosphorylation of LC20 by myosin phosphatase[15,16]. BFCF even reduced a phosphorylation of LC20 under basal levels, but no significant. So it is not likely that such decreased LC20 phosphorylation in the presence of BFCF results in vasodilation. Although it is generally accepted that the primary mechanism of smooth muscle contraction involves Ca^{2+}-dependent phosphorylation of LC20 by MLCK, additional mechanism are also believed to affect the contractile state of smooth muscle. The involvement of PKC activation in smooth muscle contraction originated from the observation that phorbol esters, known to activate PKC, induce slow sustained contractions in several types of vascular smooth muscle strips[17-20]. Signal transduction via PKC is closely regulated by its subcellular localization. In response to activation of cell-surface receptors, PKC is directed to the plasm membranes[21], resulting from changes in the cellular levels and intracellular localization of Ca^{2+} and diacylglycerol(DAG). In addition, activation of Ca^{2+}-dependent isozymes of PKC(α and/or β) are associated with phenylephrine-induced contraction in portal vein[9].

In the present experiment, phenylephrine induced translocation of PKC α from the cytosol to the cell membrane. However, the PKC α translocation were inhibited in the presence of BFCF in all concentrations.

On the other hand, it has been known for some time that agonist-induced contractions are often associated with Ca^{2+} sensitization[22]. The major mechanism of Ca^{2+} sensitization of smooth muscle contraction is through inhibition of the smooth muscle myosin phosphatase(MP) that dephosphorylates the regulatory light chin in smooth muscle. Until now, the subcellular distribution of the MYPT1 and PP1c subnits has been studied only in cultured rat aortic cells[23]. We have recently demonstrated that the subcellular distribution of MP is agonist-specific in fully differentiated smooth muscle cells[5]. In this study, MYPT1 appeared to be distributed throughout the cell with phenylephrine. In case of PP1c, first it goes cell membrane by 5 min and then redistributed throughout the cells. This result consistent with our previous experiments[5]. However, in the presence of BFCF, the membrane targeting of PP1c was inhibited at 5 min. The isolated PP1c has reduced phosphatase activity toward phosphorylated myosin[24]. Thus, BFCF continues to keep both subnits distributing in the cytosol. These results provide that BFCF might be increase reduced phosphatase activity through inhibition of both MYPT1 and PP1c dissociation and PKC α translocation.

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