Apelin Stimulates Myosin Light Chain Phosphorylation in Vascular Smooth Muscle Cells

Tatsuo Hashimoto, Minoru Kihara, Junji Ishida, Nozomi Imai, Shin-ichiro Yoshida, Yoshiyuki Toya, Akiyoshi Fukamizu, Hitoshi Kitamura, Satoshi Umemura

Objective—Physiological roles of apelin and its specific receptor APJ signaling were investigated in vascular smooth muscle cells (VSMCs). The present study determined whether apelin activates myosin light chain (MLC), a major regulatory event in initiating smooth muscle contraction.

Methods and Results—To assess MLC activation, we performed Western blot and immunohistochemical studies using an antibody against the phospho-MLC. In VSMCs, apelin induces the phosphorylation of MLC in a concentration-dependent manner with a peak at 2 minutes. Pretreatment of VSMCs with pertussis toxin abolishes the apelin-induced phosphorylation of MLC. Inhibition of protein kinase C (PKC) with GF-109203X markedly attenuated the apelin-induced MLC phosphorylation. In addition, methylisobutyl amiloride, a specific inhibitor of the Na⁺/H⁺ exchanger (NHE), and KB-R7943, a potent inhibitor for the reverse mode of the Na⁺/Ca²⁺ exchanger (NCX), significantly suppressed the action of apelin. In wild-type mice, apelin phosphorylates MLC in vascular tissue, whereas it had no response in APJ-deficient mice by Western blot and immunohistochemistry. Apelin-induced phosphorylation of MLC was accompanied with myosin phosphatase target subunit phosphorylation.

Conclusions—These results provide the first evidence to our knowledge for apelin-mediated MLC phosphorylation in vitro and in vivo, which is a potential mechanism of apelin-mediated vasoconstriction. (Arterioscler Thromb Vasc Biol. 2006;26:1267-1272.)

Key Words: apelin  ■  APJ  ■  myosin light chain  ■  myosin phosphatase target subunit  ■  vasoconstriction

Apelin was recently identified from bovine stomach as an endogenous ligand for APJ, a putative receptor protein related to the angiotensin-type 1 receptor (AT1). Despite sharing 31% amino acid sequence homology with AT1, APJ does not display specific binding for angiotensin II. Apelin and APJ are distributed in various tissues including the heart, blood vessels, brain, and gastrointestinal tract, although the physiological role of apelin and APJ is not well understood.

In the vascular system, apelin and APJ are known to be expressed in endothelium and smooth muscle cells (VSMCs). Histological studies in rat show that the VSMCs of the medial layer of the aorta and pulmonary artery display intense staining for APJ receptor-like immunoreactivity. In spontaneously hypertensive rats, APJ and apelin expression in both heart and aorta were markedly depressed compared with Wistar-Kyoto rats, which suggests a pathophysiological role for APJ and apelin in vascular disease.

The action of apelin in blood pressure regulation is controversial. Although the systemic administration of apelin produces transient hypotension in anesthetized and conscious rats, a potent vasoconstrictive effect of the peptide has been demonstrated in the isolated human saphenous vein. These results suggest that the apelin-APJ has biphasic effects on blood vessels via the endothelium-mediated vasodilatation and VSMCs-dependent vasoconstriction. Our previous study on murine endothelium demonstrated endothelial nitric oxide synthase phosphorylation by the specific apelin-induced stimulation. At the present time, however, the intracellular mechanisms of apelin have not been elucidated in VSMCs. Here, we focus on VSMCs to investigate apelin/APJ-mediated intracellular signal transduction mechanisms with particular reference to myosin light chain (MLC) phosphorylation. MLC is a major regulatory molecule for smooth muscle contraction.

Methods

Cell Culture

VSMCs from the thoracic aorta of 4-week-old male Wistar rats were prepared by the explant method and cultured in Dulbecco’s modified Eagle’s Medium as described previously. VSMCs multiplying in a medium containing FBS rapidly lose their contractile features and become “synthetic” cells. Thus, we confined our studies to early...
passage cells (third to the sixth passage). Subconfluent VSMCs were used in the following experiments. Cells at subconfluence were serum deprived for 24 hours and then stimulated as indicated. After the desired incubation period, cells were rinsed with ice-cold phosphate-buffered saline and then lysed and sonicated. The experiments were conducted under the guidelines for animal experiments set by the Animal Experiment Committee of the Yokohama City University School of Medicine.

**Reverse-Transcriptase Polymerase Chain Reaction**

Total RNA was isolated from the VSMCs by the acid guanidinium thiocyanate-phenol-chloroform extraction method and reverse-transcriptase polymerase chain reaction (RT-PCR) for APJ and GAPDH mRNA was performed;16-17 0.3 μg of the sample RNA was reverse-transcribed using SuperScript III™ reverse transcriptase (Invitrogen, Ontario, Canada) and oligo(dt)12 to 18 primers. APJ cDNA was amplified in PCR reactions using the following primers: sense, 5′-ATG GAA GAT GAT GGT TAC AAC TAC T-3′; antisense, 5′-CGA AAC CAC GTC CAG ACG ACC-3′. The PCR products were size fractionated on 7.5% polyacrylamide gels, stained with ethidium bromide, and then photographed. APJ-deficient mice were generated as described previously,9 and the thoracic aorta of 10-week-old male mice were examined.

**Tissue Preparation**

Thoracic aortae were carefully isolated from mice after chloroform anesthesia and immediately immersed in ice-cold modified Kreb’s solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH2PO4, 1.17 mmol/L MgSO4, 2.5 mmol/L CaCl2, 5.0 mmol/L HEPES, 11.1 mmol/L D-glucose, pH 7.4). Fat and connective tissues were then removed from the preparation. Endothelium was removed by gently rubbing the intima with a cotton swab to avoid the endothelium-dependent effect. Each aorta was equilibrated for 1 hour at 37°C in modified Kreb’s solution. Thereafter, the aortae were treated with or without apelin (10-6 mol/L) for indicated periods and rinsed with ice-cold phosphate-buffered saline and then lysed and sonicated.

**Western Blot**

Western blot was performed to determine APJ expression and the levels of phosphorylation of MLC and myosin phosphatase target subunit (MYPT) in VSMCs and vascular tissue. Soluble proteins were fractionated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, Mass). APJ protein was detected by immunoblotting with rabbit antibodies directed against mouse APJ (1:200).18 Activation of MLC by phosphorylation on Thr 18 and/or Ser 19 was revealed by immunoblotting with rabbit polyclonal antibodies against the phospho-MLC (1:1000; Santa Cruz Biotechnology). Total MLC protein was detected using rabbit polyclonal antibodies against total MLC (1:1000; Santa Cruz Biotechnology), whereas total MYPT protein was detected using rabbit polyclonal antibodies against total MYPT (1:200; Santa Cruz Biotechnology). Immunoreactive bands were visualized with specific antibodies using ECL plus reagent (Amersham Biosciences, Piscataway, NJ), and the images were analyzed by densitometry using Scion Image Software (Scion Corporation, Frederick, Md).21,22 Pertussis toxin (PTX) (Seikagaku Co, Tokyo, Japan), GF109203X (Tocris, Ellisville, Mo), U-73122 (Calbiochem, Darmstadt, Germany), methyl-isobutyl amidolride (MIA) (Sigma-Aldrich, St Louis, Mo), and KB-R7943 (Tocris) were added to the culture 24 hours, 1 hour, 1 hour, 5 minutes, and 5 minutes before [Pyr1]-apelin-13 (Peptide Institute, Osaka, Japan) stimulation, respectively.

**Immunohistochemistry**

Immunohistochemistry for formalin-fixed aorta treated with or without apelin was performed as follows. Sections of 3 μm were cut and mounted on glass slides. The endogenous peroxidase was blocked with 3% H2O2, and nonspecific binding sites were blocked with TBS containing 5% normal goat serum. Slides were then incubated with rabbit polyclonal antibody against the phospho-MLC (1:200; Santa Cruz Biotechnology) for 1 hour at room temperature. For negative controls, the primary antibody was replaced with control rabbit IgG (Santa Cruz Biotechnology). Staining was visualized with the avidin-biotin immunoperoxidase reaction using diaminobenzidine (LSAB2 Kit; DAKO, Glostrup, Denmark), and the images were analyzed by staining densitometry using Scion Image Software (Scion Corporation) as described previously.21,22

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical significance was determined by ANOVA with P<0.05 being deemed statistically significant.

**Results**

**Expression of APJ in VSMCs**

Gene expression of APJ in VSMCs was analyzed by RT-PCR. As shown in Figure 1A, the APJ mRNA was amplified using RNA prepared from cultured rat VSMCs and from the aorta of wild-type mice. No amplified product was detected using RNA prepared from the aorta of APJ-deficient mice. The APJ antibody detected a band at ~50 kDa in isolated thoracic aorta of wild-type mice (Figure 1B). There was no detectable band in the aorta of APJ-deficient mice. This is consistent with a previous report showing APJ receptor expression in HEK-293 cells stably expressing the APJ.6

**Effect of Apelin on MLC Phosphorylation in Cultured VSMCs**

Activation of MLC is regulated by phosphorylation. Ser 19 of MLC has been shown to be the primary residue phosphory-
lated by MLC kinase, with Thr 18 as a secondary site. Western blot analysis showed apelin to induce MLC phosphorylation at Thr 18 and/or Ser 19. The phosphorylation reaction reached a maximal level 2 minutes after the addition of apelin (10−7 mol/L) (Figure 2A). Furthermore, the reaction proceeded in a concentration-dependent (0 to 10−5 mol/L) manner (Figure 2B).

### Inhibitors of G Protein, Protein Kinase C, or Ion Exchangers

In the following experiments, the effects of drugs were evaluated 2 minutes after the stimulation of apelin (10−7 mol/L). To examine intracellular signal transduction mechanisms, VSMCs were pretreated with inhibitors for G protein, PKC, and ion exchangers.

As shown in Figure 3, PTX (100 ng/mL), which uncouples Gi/Go proteins from their receptors by ADP-ribosylation, abolished the apelin-induced phosphorylation of MLC. GF109203X (10−5 mol/L), a specific inhibitor of PKC, also abolished the apelin-induced phosphorylation of MLC. U-73122 (10−5 mol/L), a potent inhibitor of phospholipase C (PLC), had no influence on MLC phosphorylation, although it abolished the angiotensin II-induced phosphorylation of MLC (data not shown). In the absence of apelin, the addition of PTX, GF109203X or U-73122 did not affect MLC phosphorylation (data not shown).

MIA (10−5 mol/L), an inhibitor of the Na+/H+ exchanger (NHE), and KB-R7943 (10−5 mol/L), an inhibitor of the Na+/Ca2+ exchanger (NCX), also significantly attenuated the overall apelin-induced MLC phosphorylation (supplemental Figure I, please see http://atvb.ahajournals.org.). However, the effects of these inhibitors were not complete; densitometric analysis of 3 separate experiments revealed that the phosphorylation reaction was reduced to 73% and 81% of the control by MIA and KB-R7943, respectively. In the absence of apelin, MIA and KB-R7943 did not affect MLC phosphorylation (data not shown).

---

**Figure 2.** Time course of phosphorylation of MLC by apelin in VSMCs is shown (A). VSMCs were starved for 24 hours, stimulated with 10−7 mol/L apelin for the indicated periods of time, and then lysed and analyzed by SDS-PAGE and immunoblotting for Thr 18/Ser 19 phosphorylated MLC (A, upper panel) or total MLC protein (A, lower panel) using specific antisera. Apelin promotes a concentration-dependent phosphorylation of MLC (B). VSMCs were stimulated with varying concentrations of apelin for 2 minutes and lysates were immunoblotted as described previously. The intensity of each band on the blot was quantified by densitometric scanning, and the activities of MLC are shown as fold increases of the average from 3 independent experiments by comparison with nonstimulated controls (1.0). *P<0.05 vs nonstimulated controls.

**Figure 3.** Phosphorylation of MLC by apelin involves a PTX-sensitive protein and PKC. VSMCs were treated with PTX (100 ng/mL) for 24 hours (A), GF109203X (10−5 mol/L) for 1 hour (B) or U-73122 (10−7 mol/L) for 1 hour (C), followed by stimulation with apelin (10−7 mol/L) for 2 minutes. MLC activity was measured as described in the legend to Figure 2. The intensity of each band on the blot was quantified by densitometric scanning, and the activities of MLC are shown as fold increases of the average from three independent experiments compared with unstimulated controls (1.0). *P<0.05 vs nonstimulated controls, **P<0.05 vs apelin-stimulated groups.
Effect of Apelin on MLC Phosphorylation in Isolated Thoracic Aorta

Thoracic aortae were isolated and treated with or without apelin (10^{-6} mol/L). Western blot showed apelin to induce MLC phosphorylation. The phosphorylation reaction reached a maximal level 2 minutes after the addition of apelin (10^{-6} mol/L) (Figure 4A). The MLC phosphorylation was accompanied with MYPT phosphorylation (Figure 4B). Immunohistochemistry showed MLC phosphorylation in the medial layer of aorta from wild-type mice (Figure 5A). Positive immunostaining for phospho-MLC is highly localized in the medial layer of the aorta treated with apelin (Figure 5B). Staining area for phospho-MLC in the medial layer of aorta significantly increased in response to the apelin treatment. No such response was observed in the aorta from APJ-deficient mice (Figure 5C). Figure 5D shows phospho-MLC expression in the wild-type mice treated with angiotensin II (10^{-6} mol/L) as a positive control. In the control experiments, where the primary antibody was replaced with normal rabbit IgG, the samples were negative for phospho-MLC (Figure 5E). Removal of the endothelium was verified by HE staining (Figure 5F). These observations were quantitatively evaluated (Figure 5G). In wild-type mice, we detected 1.9 times more phospho-MLC staining area in the aorta treated with apelin than with nontreated aorta. We detected no differences in phospho-MLC staining area between nontreated wild-type mice aorta and APJ-deficient mice aorta treated with apelin. These results indicate apelin induces significant phosphorylation of MYPT and MLC in aorta via the APJ receptor.

Discussion

In the present study we have demonstrated that APJ is expressed in aortic VSMCs at the mRNA and protein level. This is consistent with a previous study, which showed the autoradiographical localization of apelin binding in the medial layer of the human coronary artery and saphenous vein13 and immunohistochemical staining for APJ in human and rat blood vessels.7 Primary mechanisms for the contraction of smooth muscle are the phosphorylation of MLC by MLC kinase and inhibition of MLC phosphatase. The transient increase in MLC phosphorylation, a biochemical index of vascular contraction, can be stimulated in vascular smooth muscle preparations by a variety of vasoactive hormones and neurotransmitters,
including angiotensin II, histamine, α-adrenergic agonists, and neuropeptide Y. Apelin is derived from a 77-amino-acid precursor, and processed to several isoforms by modifying the amino terminus. The pyroglutamylation form of apelin-13, [Pyr1]-apelin-13, has been reported to be produced in vivo in bovine colostrums and have activity at the receptor. In our experiment, apelin induced significant phosphorylation of MLC in cultured rat VSMCs in a dose-dependent fashion. The in vitro response to apelin stimulation is the same in the VSMCs of the intact arterial media. Apelin enhanced the phospho-MLC immunoreactivities in freshly isolated thoracic aortae of wild-type mice, whereas the same dose of the peptide had no effect on aortae of APJ-deficient mice. Immunohistochemical staining for phospho-MLC using wild-type mice and APJ-deficient mice strengthened that apelin induced phosphorylation of MLC. Apelin-induced phosphorylation of MLC was accompanied with MYPT activation of PKC, NHE, NCX, and MYPT. This is the first report for the potential mechanism of vasoconstriction via APJ receptors.

It has been shown that apelin/APJ signaling is coupled to PTX-sensitive G proteins (Gi or Go protein) in cultured cells. In the present study, we have demonstrated apelin-stimulated MLC phosphorylation in VSMCs in a PTX-sensitive manner. This result suggests that apelin/APJ signaling mediates Gi or Go protein to phosphorylate MLC in VSMCs. The mode of action of apelin may be like other Gi or Go protein-linked vasoconstrictors such as sphingolipids, noradrenaline, and neuropeptide Y. It has been demonstrated that PKC enhances the phosphorylation of MLC at multiple sites in smooth muscle and promotes contraction of the aorta. In our experiments, apelin-induced MLC phosphorylation was markedly attenuated by GF109203X, a specific inhibitor of PKC, suggesting that PKC activates apelin-induced MLC phosphorylation. PKC can activate various target proteins in VSMCs. Among them, NHE and NCX are known to play important roles in tonic contraction of arterial smooth muscle cells, such that activated NHE mediates the elevation of the cytosolic concentration of sodium, leading to an influx of calcium through NCX. We showed that MIA and KB-R7943 markedly reduced the MLC phosphorylation stimulated by apelin in VSMCs, suggesting the involvement of NHE and NCX in apelin-induced MLC phosphorylation. The apelin-induced increase in developed tension of isolated rat heart was partly reduced by PTX and markedly attenuated by U-73122, a potent inhibitor of phospholipase C. In our experiments, PTX significantly lowered apelin-induced MLC phosphorylation in VSMCs. However, U-73122 failed to inhibit the apelin-induced phosphorylation of MLC. The coupling of G protein with APJ appears to be different in VSMCs and heart. This is the first report to demonstrate the potential mechanism of apelin-induced vasoconstriction. Our findings are consistent with a previous report showing that apelin potently contracts the human saphenous vein. However, we and others reported that blood pressure is transiently lowered by the systemic administration of apelin via APJ, which involves the activation of the nitric oxide/L-arginine system. Because APJ-mediated apelin stimulation phosphorylates endothelial nitric oxide synthase in cultured endothelial cells, the apelin-induced vasodilatation may depend on the endothelial cells. Both VSMCs and endothelial cells express APJ, suggesting a biphasic action. From a pathophysiological point of view, it might be speculated that apelin functions as a vasopressor in damaged vasculature (eg, atherosclerosis). A recent report showed that insulin upregulates apelin mRNA expression in adipocytes and both plasma apelin and insulin levels were significantly higher in obese patients. Additional studies will determine whether apelin is involved in the obesity-associated vascular damage.

In conclusion, we have demonstrated that APJ is expressed in VSMCs, and that apelin induces the phosphorylation of MLC in vitro and in vivo. The apelin-induced MLC phosphorylation may involve PTX-sensitive G proteins, and activation of PKC, NHE, NCX, and MYPT. This is the first report for the potential mechanism of vasoconstriction via APJ in VSMCs.

Acknowledgments
This study was supported by grants from the 21st Century COE Program, from the Japan Society for the Promotion of Science Grant (JSPS), a grant-in-aid for Scientific Research from the JSPS (2002, 13670735) (2004, 16590704), a grant-in-aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (2004, 16709425), and from the Yokohama Foundation for Advancement of Medical Science. We acknowledge Dr Akihide Ryo, Department of Molecular Pathology, Emi Maeda, and Hiroko Morinaga, Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine and School of Medicine for scientific discussions, encouragement, and technical assistance.

References


Apelin Stimulates Myosin Light Chain Phosphorylation in Vascular Smooth Muscle Cells

Tatsuo Hashimoto, Minoru Kihara, Junji Ishida, Nozomi Imai, Shin-ichiro Yoshida, Yoshiyuki Toya, Akiyoshi Fukamizu, Hitoshi Kitamura and Satoshi Umemura

*Arterioscler Thromb Vasc Biol.* 2006;26:1267-1272; originally published online March 23, 2006;
doi: 10.1161/01.ATV.0000218841.39828.91

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/26/6/1267

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/
Fig. I.
Figure I.

Phosphorylation of MLC by apelin involves NHE and NCX. VSMCs were treated with MIA (10^{-5} M) (A) and KB-R7943 (10^{-5} M) (B) for 5 min, followed by stimulation with apelin (10^{-7} M) for 2 min. MLC activity was measured as described in the legend to Figure 2. The intensity of each band on the blot was quantified by densitometric scanning, and the activities of MLC are shown as fold increases of the average from three independent experiments compared with unstimulated controls (1.0). *P<0.05 vs. non-stimulated controls, **P<0.05 vs. apelin-stimulated groups.