(Form4-2)

JOINT RESEARCH PROJECT

FINAL REPORT
For Japan-Korea Joint Research Project

AREA	1. Mathematics & Physics 2. Chemistry & Material Science 3. Biology 4. Informatics & Mechatronics 5. Geo-Science & Space Science ⑥. Medical Science 7. Humanities & Social Sciences

1. Research Title:

Regulation of vascular contraction by PI3KC2α and targeting it for treatment of hypertension

2. Term of Research: From July 1, 2010 To March 31, 2012

3. Total Budget

a. Financial Support by JSPS: Total amount: 2,400 thousand yen

1st Year 1,200 thousand yen 2nd Year 1,200 thousand yen

3rd Year 0 thousand yen

b. Other Financial Support : Total amount: 0 thousand yen

4. Project Organization

a. Japanese Principal Researcher	
Name	Yoh Takuwa
Institution / Department	Kanazawa University, Institute of Medical, Pharmaceutical, and Health Sciences/ Department of Molecular Vascular Physiology
Position	Professor
b. Korean Principal Researcher	
Name	InKyeom Kim
Institution / Department	Kyungpook National University School of Medicine/ Department of Pharmacology
Position	Professor

c. List of Japanese-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Yasuo Okamoto	Kanazawa University, Institute of Medical, Pharmaceutical, and Health Sciences/ Department of Molecular Vascular Physiology	Associate Professor
Kazuaki Yoshioka	Kanazawa University, Institute of Medical, Pharmaceutical, and Health Sciences/ Department of Molecular Vascular Physiology	Assistant Professor

d. List of Korean-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Young Mi Seok	Kyungpook National University School of Medicine/ Department of Pharmacology	Assistant Professor
Hae Ahm Lee	Kyungpook National University School of Medicine/ Department of Pharmacology	Post-doctoral fellow
Su Bun Jeon	Kyungpook National University School of Medicine/ Department of Pharmacology	MD-PhD student
Inji Baek	Kyungpook National University School of Medicine/ Department of Pharmacology	Research assistant
Dong-Youb Lee	Kyungpook National University School of Medicine/ Department of Pharmacology	Graduate student

5. Number of Exchanges during the Final Fiscal Year*

a. from Japan to Korea

*Japanese fiscal year begins April 1.

Name	Home Institution	Duration	Host Institution

For Final Fiscal Year(FY2011)	For Final Fiscal Year(FY2011)
Total: 0 persons	Total: 0 man-days

Numbers of Exchanges during the past fiscal years

FY2009: Total 0 persons
FY2010: Total 1 persons

b. from Korea to Japan

Name	Home Institution	Duration	Host Institution
InKyeom Kim	Kyungpook National University	January 9, 2012~ January 11, 2012	Kanazawa University

For Final Fiscal Year(FY2011)	For Final Fiscal Year(FY2011)
Total: 1 persons	Total: 3 man-days

Numbers of Exchanges during the past fiscal years

FY2009: Total 1 persons
FY2010: Total 1 persons

6. Objective of Research

Ca^{2+} plays a pivotal role in vascular smooth muscle (VSM) contraction. It has been recognized for more than 20 years that Ca^{2+} activates myosin light chain kinase (MLCK) to induce 20 kDa myosin light chain (MLC) phosphorylation and thereby contraction. We recently discovered the second target molecule of Ca^{2+} in VSM contraction, i.e. small G protein Rho, which inhibits MLC phosphatase (MLCP) through Rho kinase. This discovery implys that Ca^{2+} increases MLC phosphorylation and contraction through both MLCK activation and MLCP inhibition (two branch model, Fig. 1). Moreover, we found that class II phosphoinositide 3-kinase (PI3K) α-isoform (PI3K-C2α), the function of which remained nearly unknown in mammals, mediates Ca^{2+}-dependent Rho activation and MLCP inhibition. Although we established the essential role of PI3K-C2α in isolated VSM, the in vivo role of PI3K-C2α-Rho-Rho kinase-MLCP signaling pathway is still totally unknown.

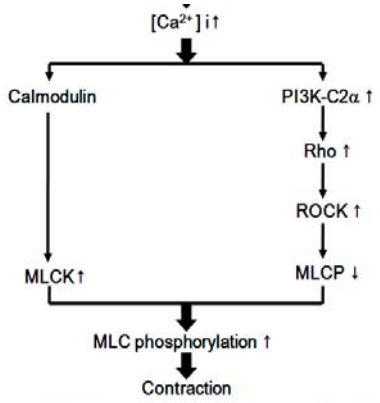

Fig. 1. Novel Ca^{2+}- and PI3K-C2α-dependent Rho activation and MLCP inhibition, which together with Ca^{2+} -dependent MLCK activation induces efficient phosphorylation of MLC and contraction.

The aims of this project are to study a role of PI3K-C2α in physiological regulation of the blood pressure and the development of pathologically elevated blood pressure, i.e. hypertension. For the evaluation of a physiological role of PI3K-C2α in the blood pressure regulation, we will take advantage of conditional smooth muscle-specific PI3K-C2α-knockout (KO) mice that we originally generated. For the evaluation of a role of PI3K-C2α in hypertension, we employ a rat hypertension model, spontaneously hypertensive rats (SHRs). Since a Ca^{2+} channel blocker effectively lowers blood pressure in various types of hypertension including SHRs and Ca^{2+} activates PI3K-C2α, it is very likely that PI3K-C2α plays an important role in hypertension. We analyze a role of Ca^{2+}- PI3K-C2α-Rho-Rho kinase-MLCP signaling pathway by employing the originally developed analytic methods for the PI3K-C2α and Rho pathways, and test the effects of natural ingredient libraries from plants that Dr. Kim's group made. These investigations will promote our understanding molecular mechanisms behind hypertension, and open the new avenue for developing a new anti-hypertensive drug.

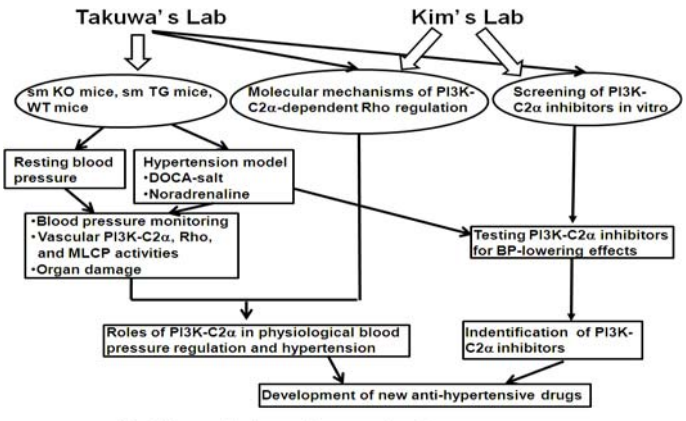

Fig. 2. Research plans and co-operations between two groups

7. Methodology

For studying a role of PI3K-C2α in the physiological regulation of blood pressure, we used smooth muscle-specific conditional PI3K-C2α-KO mice. Mice with smooth muscle specific deletion of PI3K-C2α were generated by mating PI3K-C2α$^{flox/flox}$ X Sm22-Cre transgenic mice (manuscript in preparation).

The animal hypertension model of spontaneously hypertensive rats (SHRs) and the control normotensive and Wister Kyoto (WKY) rats were used. The blood pressure was non-invasively determined by a tail cuff method using Softron BP98A (Softron BP98A, Softron Co Ltd, Tokyo). Wortmannin (WMN), nicardipine and hydralazine were injected via the tail vein over 1 min.

The amounts of GTP-bound RhoA (GTP-Rho) was determined as follows: quickly frozen aortic tissues were homogenized and the supernants after centrifugation were incubated with GST-Rhotekin immobilized onto glutathione-Sepharose 4B beads. The beads were washed and solubilized with Laemmli's SDS sample buffer, followed by separation on a acrylamide gel and Western analysis using anti-RhoA antibody.

Phosphorylation of the regulatory subunit MYPT1 of myosin phosphhatase was determined as follows: quickly frozen aortic tissues were homogenized and separated on SDS-PAGE, followed by Western blot analysis using either phospho(Thr^{853})-MYPT1 or phospho(Thr^{696})-MYPT1 specific antibodies and anti-total MYPT1 antibody. The amounts of phospho(Thr^{853})-MYPT1 quantified by densitometry were normalized for total amounts of MYPT1.

Vascular activities of PI3K-C2α and PI3K-p110α were determined as follows: quickly frozen aortic tissues were homogenized, and the supernatants were subjected to immunoprecipitation using specific polyclonal anti-PI3K-C2α antibody. The anti-PI3K-C2α immunoprecipitates were incubated with the kinase assay buffer containing [γ-^{32}P]ATP and phosphatidylinositol (PI). The reaction was terminated and extracted lipid fraction was separated by thin layer chromatography. Radioactivity in the spot corresponding to PI 3-phosphate was quantitated by using FujiBAS Bioimage analyzer 2000 (Fuji, Tokyo, Japan), normalized for the PI3K-C2α protein amount in each sample.

Plants *Schisandra chinensis* fruits, from which ingredients were isolated were, collected from Mungyeong, Korea. The chemical structure of GA was verified by liquid chromatography–mass spectrometry (LC–MS, Bruker BioApex FT mass spectrometer) and NMR analysis (Bruker DRX 400 spectrometer), a JASCO DIP-370 digital polarimeter, a Bruker DRX 400 spectrometer, and a Bruker FT mass spectrometer.

Isometric tension of vascular smooth muscle was determined by using an isometric force transducer (Grass FT03C, Quincy, Mass., USA) in an organ bath. With animals under anesthesia (sodium pentobarbital 50 mg kg^{-1} i.p.), the thoracic aorta was immediately excised and immersed in an ice-cold, modified Krebs solution composed of (in mM) NaCl, 115.0; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and dextrose, 10.0. The aorta was cleaned of all adherent connective tissue on wet filter paper, soaked in the Krebs-bicarbonate solution and cut into four ring segments (4 mm in length) as described by Seok' method (Seok et al. 2006). In order to investigate the direct effect of GA in vascular smooth muscle, the rings were denuded of endothelium by gently rubbing the internal surface with a forcep edge. Two stainless-steel triangles were inserted through each vessel ring. Each aortic ring was suspended in a water-jacketed organ bath (22 ml) maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.