日本財団 The Nippon Foundation 助成事業

財団法人　日中医学協会

2009年度共同研究等助成金報告書－在留中国人研究者－

2010年　3月3日

財団法人　日中医学協会　御中

貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

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1．助成金額：　600,000　円

2．研究テーマ

新しく見つけた血管平滑筋収縮制御の解析

3．成果の概要

血管は主に平滑筋より成り立ち、アクトミオシン系蛋白質が収縮力を生み出している。その制御はミオシン軽鎖キナーゼ(MICKと略す)によって行われ、ミオシン軽鎖のリン酸化によるものと考えられている。しかし、近年の技術革新によりこの筋書き通りではなく、リン酸化によらなくても収縮が起こる実例が分かってきた。リン酸化を完全に阻害した条件で細胞遊走をおこなわせて、誘引物質にたいする効果を調べた。

4．研究業績

(1)学会における発表　　㊙・有(学会名・演題)

(2)発表した論文　　無・㊒(雑誌名・題名)

Xie C, Zhang Y, Wang HH, Matsumoto A, Nakamura A, Ishikawa R, Yoshiyama S, Hayakawa K, Kohama K, and Gao Y. Calcium regulation of non-kinase and kinase activities of recombinant myosin light-chain kinase and its mutants. *IUBMB Life* 61: 1092-1098, 2009

—日中医学協会助成事業—

アラキドン酸に対する血管平滑筋の遊走：

ミオシン軽鎖キナーゼのリン酸化が阻害される条件での検討

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要　旨

Arachidonic Acid Induces Migration of Vascular Smooth Muscle Cells Under the Conditions Where Phosphorylation of Myosin Light Chain is Abolished. Migration of vascular smooth muscle cells (VSMCs) plays an important role in vascular development as well as pathogenesis of atherosclerosis. Studying the mechanism involved in VSMCs migration and ultimately finding a way to block the migration of VSMCs in the development of vascular lesion, has been a focus of research. Activation of myosin II by phosphorylating its' myosin light chain (MLC) is widely accepted to be a major, regulated determinant of producing contractile forces in cell motility. To reveal the mechanism for un-phosphorylated myosin to cause migration, we tested the effects of several chemoattractants on migration of SM3 cells in presence of ML-7, which prevented the phosphorylation of MLC. Among the several chemoattractants, we report here migration of VSMCs is induced by arachidonic acid (AA) while phosphorylation level of MLC is totally abolished by ML-7. To evaluate the migratory activity of SM3 cells toward AA in a quantitative way, we used various concentrations of AA as chemoattractants in boyden chamber assay. SM3 cells migrated toward AA maximally at 20μM, although intracellular phosphorylation of MLC was totally abolished. Formations of filopodia and lamellipodia were observed by immunofluorescence staining in this condition, indicating the involvement of actin in the migration. However, blebbistatin, specific inhibitor to myosin II head domain, blocks this migration, suggesting the motor activity of myosin II is producing the force of migration. We studied the signaling pathway of this AA-induced migration in which the MLC phosphorylation was blocked by ML-7. PTX inhibited this migration, suggesting the role of G protein coupled receptors (GPCR). Since intracellular calcium wasn't altered in this condition, we further investigated the down-stream signaling factors which were intracellular calcium increase-independent. We used specific inhibitors or siRNA to study the role of several known down-stream signaling factor of GPCR, finding that membrane translocation of PLCβ2, phosphorylation of PKCε, phosphorylation of MAPKs (ERK, p38 and JNK) occurred successively after the stimulation of AA. It was possible that AA interacted GPCR as its ligand, triggering following signal pathway. However, we found that AA increased the secretion of 15-HETE in cell media and 15-HETE also induced this migration, indicating that 15-HETE, a LOX metabolite of AA, is an equally possible ligand. We also indicated that AA penetrated the cellular membrane of VSMCs followed by the stimulation of the ATPase activity of myosin II with

unphosphorylated MLC. Thus, we propose a new signal pathway of the migration of VSMCs which was independent of MLC phosphorylation.
Key words: Vascular smooth muscle cells, Myosin light chain, Phosphorylation, Arachidonic acid, Migration

緒 言

Migration of vascular smooth muscle cells (VSMCs) plays an important role in vascular development as well as pathogenesis of atherosclerosis. During atherogenesis, migration of VSMCs from media to intima is believed to contribute intimal thickening (*1*). Studying the mechanism involved in VSMCs migration and ultimately finding a way to block the migration of VSMCs in the development of vascular lesion, has been a focus of research(*2*). In cells migratory process, different forces are needed to perform cellular dynamic behaviors such as protrusive forces used to extend lamellipodia, and traction forces to propel the cell body forward (*3*). Regulation of the contractile forces coupled with cellular adhesion, protrusion, and actin organization depends on activated myosin II-based motors, in which the phosphorylation of the myosin light chain (MLC) is widely believed to be essential (*4*). Activation of myosin II are attributed for the phosphorylation of MLC which is regulated by two main distinct mechanisms including Ca^{2+}-dependent activation of myosin light chain kinase (MLCK) and Ca^{2+} -independent inhibition of MLCP by Rho Kinase (ROCK) (*5*). The generation of contractile force in migration of VSMCs was thought to be similar to that of regulating smooth muscle contraction (*6*). Interestingly, serum and PDGF induced VSMCs contraction was uncoupled to elevation of phosphorylation of MLC (*7*). The production of contractile forces may not through the elevation in phosphorylation level of MLC (*8*). In regard to migration, increase of MLC phosphorylation was not involved in PDGF-induced VSMCs migration (*9*). VSMCs migration may be regulated not only by an MLC phosphorylation-dependent pathway, but also an MLC phosphorylation-independent pathway. Blebbistatin, specific inhibitor to myosin II, blocks both MLC phosphorylation-dependent and independent migration of VSMCs, suggesting the essential role of motor activity of myosin in cell migration (*10*).

対象と方法

In the whole study, we used SM3 cells, which is a vascular smooth muscle cell line established from rabbit aorta arterial smooth muscle. The block-it RNAi designer (Invitrogen) was used to design a short hairpin RNA molecules (shRNA) specific to PLC-β2 (5'-GAACAGAAGTTACGTTGTC-3'). The ds oligos were transfected into SM3 cells by using Lipofectamine2000 (Invitrogen). Migration of SM3 cells was assayed by the Boyden chamber method. Cells were lysed followed by western blot. In some cases, membrane fraction got subcellular fractionation purification was used. Phosphorylation of MLC was detected with glycerol-PAGE followed by Western blot. Formation of filopodia and lamellipodia were visualized by immunofluorescence staining followed by observation with confocal microscopy (Bio-Rad). $[Ca^{2+}]_i$ was measured as previously described(*11*). ELISA was used to assay the concentration level of 15-HETE in media. Statistical analysis was performed by one-way ANOVA test using Sigma Stat v.3.1. A value of $p<0.05$, $p<0.01$ was considered to be statistically significant.

結　果

As shown in Fig. 1, SM3 cells migrated toward AA maximally at 20 μM. The migration of SM3 cells toward AA (Fig. 1A) was hardly affected by ML-7, although intracellular phosphorylation of MLC was totally abolished (Fig. 1B). Formation of filopodia and lamellipodia as stained by the antibodies to α-actin and β-actin were clearly detected after the stimulation of 20 μM AA in presence of 20 μM ML-7 (Fig. 1 C), indicating the involvement of actin in the migration. Blebbistatin inhibited the AA induced migration of ML-7-treated SM3 cells in a dose-dependent way with IC50=40 μM, indicating the myosin-driven nature (Fig. 1 D).

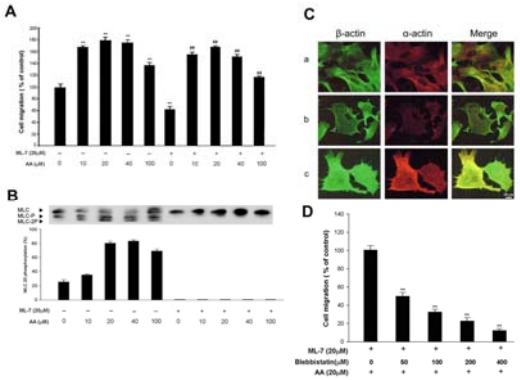

Fig. 1

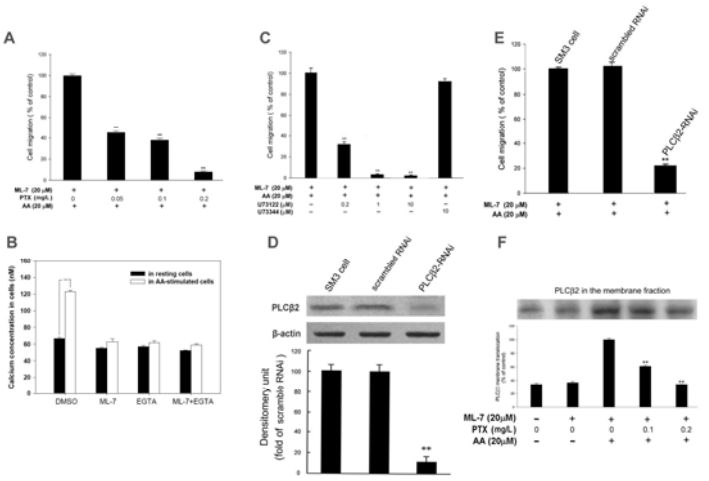

Fig. 2

Then, we carried out the analysis of signal transduction of this migration. We allowed SM3 cells to migrate in the presence of PTX, an inhibitor of a trimerc Gi protein, together with ML-7. Inhibition of the migration was observed in PTX above 0.05 mg/L(Fig. 2A). We measured $[Ca^{2+}]_i$ in the condition. When 20 μM ML-7 existed in the buffer, the increase in $[Ca^{2+}]_i$ was not observed(Fig. 2B). This migration was antagonized by U-73122 in a dose-dependent manner, indicating PLC was involved in the signal transduction pathway (Fig. 2C). To clarify the role of PLCβ2 in the migration, we designed the interfering RNA (RNAi) for PLCβ2 to decrease the expression of PLCβ2 as detected by antibody against PLCβ2 (Fig. 2D). The migration of the SM3 cells treated by PLCβ2 RNAi decreased remarkably as compared with the migratory activity of SM3 cells treated with scrambled RNAi (Fig. 2E). We further investigate the role of PLCβ2 translocation in this migration of smooth muscle cells as shown in Fig. 2F.

As shown in Fig. 3A, staurosporine, an inhibitor of PKC, depressed this migration. We further examined the effect of PKCε inhibitory peptide. Fig. 3B showed that the migration

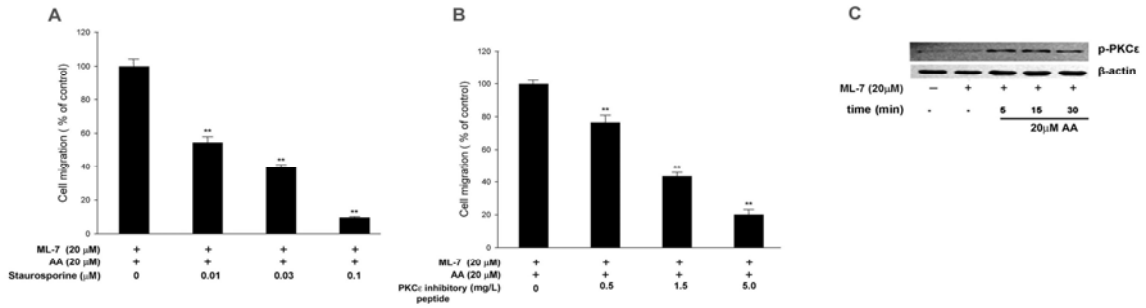

Fig. 3

was inhibited by the peptide. Accordingly, an active form of PKCε was detected by the antibody against phosphorylated PKCε, finding that PKCε was phosphorylated time-dependently in response to stimulation by AA (Fig. 3C).

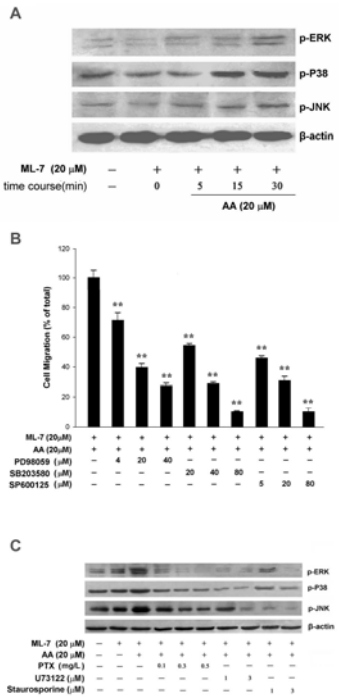

Fig. 4

Next, we confirmed the roles of MAPKs in this migration. As shown in Fig. 4A, ERK, p38 and JNK were phosphorylated after the AA stimulation as examined in the presence of ML-7. SB203580, PD98059 and SP600125 inhibited this migration (Fig. 4B). We examined the effects of inhibitors, i.e., PTX for Gi, U73122 for PLC and staurosporine for PKC, on the phosphorylation of the MAPKs. Our results indicated that ERK, p38 and JNK signal pathways were all subjected to the Gi followed by PLC signaling(Fig. 4C). ERK and JNK signaling pathways, but not p38 were under the PKC signaling. We speculate that PLC directly regulates the phosphorylation of p38.

Then, we hypothesized that AA metabolic pathways of Cox, Lox and p450 may play a role in this signaling. Only the Lox inhibitor of NDGA inhibited this migration, indicating the role of Lox metabolites (Fig. 5A). We found AA stimulated the 15-HETE secretion in media(Fig.5B). As shown in Fig 5C, we found that it could induce the migration of SM3 cells in the presence of ML-7, confirming 15-HETE mediated the migration of SM3 cells toward AA. We recently published that AA binds to myosin II heads to stimulate the ATPase activity of myosin II of which MLC remains unphosphorylated(*12*). Fig. 5C confirmed the stimulatory effect. However, the effect of 15-HETE was much lower stimulatory effect.

考　察

Production of contractile force to induce migration may be regulated by a MLC phosphorylation-independent way(*9*). The present report confirmed this possibility. AA is a well-known mediator of atherosclerosis(*13*). We proposed in this paper that the GPCR that is specific for 15-HETE should be most convincing among eicosanoid receptors. PTX inhibited the abnormal migration of VSMCs towards AA, suggesting the role of GPCRs in this migration. The role of PLCβ2 in this abnormal migration is in coincidence to previous report(*14*). We reckoned that the activation of PLCβ2 by AA was regulated by Gi protein, implying that Gi protein located in the upstream of PLCβ2. Among more than ten isoforms of PKC, PKCε were closely linked

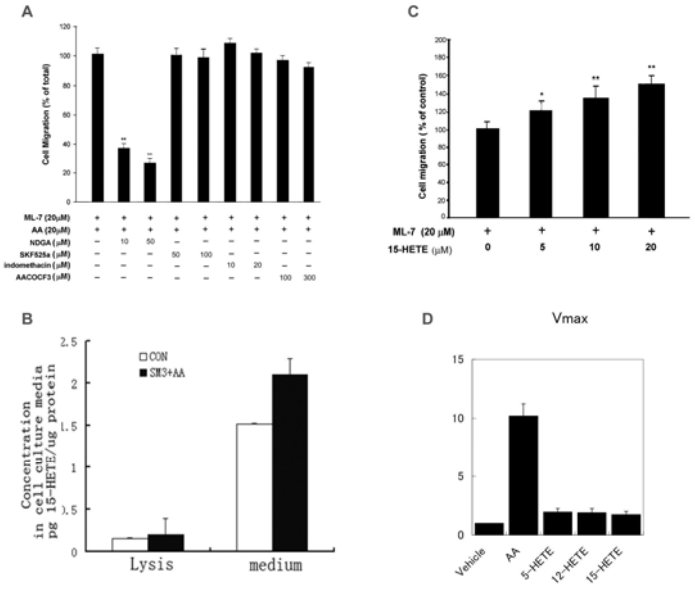

Fig. 5

to VSMCs migration(*15*).We confirmed the essential role of PKCε in this abnormal migration by specific PKCε inhibitory peptide. In short, although increase of $[Ca^{2+}]_i$ and phosphorylation of MLC were blocked by pretreatment of ML-7, our results suggested that GPCR, PLCβ and PKCε transduction pathway were activated by AA. Our results suggested activities of MAPKs were mediated by Gi protein, PLCβ and PKCε. To investigate whether other cellular factors participate in the signal pathway of this migration, we further tested the effects of tyrphostin AG112, Herbimycin, wortmannin and Y27632 and found any of the them did not inhibit the AA stimulated migration of SM3 cell with pretreatment of ML-7, excluding the signal pathways of protein tyrosine kinase, phosphoinositide kinase-3 and Rho kinase in the migration without phophorylation of MLC In summary, AA induced the migration of smooth muscle cell SM3 when phosphorylation of MLC was inhibited by ML-7. AA metabolites via Lox, 15-HETE, mediated this effect on migration. The successive signaling pathways we hypnotized were: Gi → PLCβ2 → PKCε →MAPKs. This "unusual" migration may contribute to the understanding of the vascular pathology.

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作成日：2010 年 3 月 1 日